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Intestinal Adaptation in Pediatric Short Bowel Syndrome – Controlled Assessment of Duodenal Mucosa after Extensive Bowel Resection

Galina Sanaksenaho

ACADEMIC DISSERTATION

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To the patients

ABSTRACT

Background. Although extensively studied in experimental models, human data on intestinal adaptation in short bowel syndrome (SBS) is very limited.

Aim of the study. We studied mucosal homeostasis of the duodenum in children with intestinal failure (IF) both during parenteral nutrition (PN) and after achieving intestinal autonomy by weaning off PN in relation to controls.

Patients and methods. In total, 58 patients with IF aged from 0.5 to 23 years and 43 controls matched for age and gender without intestinal pathology were included. Duodenal biopsies obtained during clinically indicated gastroscopies were collected. In patients, the median duration of PN in the first study was (I) 1.2 (IQR 0.6-5.1) years, in the second study (II) 1.4 (0.7-6.5) years and the third study (III) 0.9 (0.4-2.0) years. The remaining small bowel length for studies I-III was 49 (29-101) cm, 33 (12-60) cm and 47 (30-60) cm, or 26 (17-48) %, 20 (9-22) % and 29 (19-43) % of expected, respectively. Of the patients in each study, (I) 27 (II) 6 and (III) 16 had the ileocecal valve in bowel continuity.

We evaluated duodenal disaccharidase activities, inflammation, structural mucosal hyperplasia, proliferation, apoptosis, epithelial barrier function and nutrient transport by using the glucose oxidase method, histology, immunohistochemistry, and quantitative real-time polymerase chain reaction (qPCR) for 52 genes. Dilatation of the small bowel was assessed from a contrast small bowel series.

Results. Activities of maltase and sucrase were 1.6 times lower and mucosal inflammation more frequent (22% vs 3%) in patients on current PN in comparison with controls ($P<0.05$ for both). Disaccharidase activities in patients who had weaned off PN were comparable with controls. Follow-up time after intestinal resection correlated positively ($r=0.448$ and $r=0.369$), and the length of the remaining small bowel inversely ($r=-0.337$ and $r=-0.407$), with maltase and sucrase activities after weaning off PN ($P<0.05$ for all).

In the evaluation of mucosal morphology, proliferation, and apoptosis, PN-dependent and weaned off patients showed similar results to controls. The percentage the remaining small bowel length inversely correlated with villus height (r=-0.397, P=0.022). Compared to controls, SBS patients showed a statistically significant increase in mucosal mRNA expression of Transforming growth factor *(TGF)β2* (PN-dependent patients P=0.035, weaned off patients P=0.006) and Caveolin1 (*CAV1)* (PN-dependent patients P= 0.016 and weaned off patients P=0.001). In addition, compared to controls, patients on PN demonstrated elevated mRNA expression of Claudin 1 (*CLDN1,* P=0.044), Mucin 2 (*MUC2,* P=0.044) and Glucose transporter, GLUT1 (*SLC2A1*, P=0.035) and decreased expression of NLR family CARD containing 4 (*NLRC4*, P=0.021). Weaned-off patients showed increased histologic inflammation of the lamina propria (P= 0.033) along with elevated Tumor necrosis factor (*TNF*, P=0.027) and *TGF-β2* (P=0.006) mRNA expression in comparison with control individuals. Pathologic small bowel dilatation was associated with shorter crypts (P=0.045) and decreased mRNA expression of Interleukin *(IL)6* (P=0.008), while bowel dilatation correlated negatively with the expression of *IL6* (r=-0.609, P=0.004), proliferation marker genes Proliferating cell nuclear antigen (*PCNA*, r=-0.439, P=0.046) and Marker of proliferation Ki-67 (*MKI67*, r=-0.625, P=0.002). Loss of the ileocecal valve upregulated mRNA expression of Toll-like receptor 4 (*TLR4,* P=0.037), *TGFB1* (P=0.043) and *CAV1* (P=0.025), apoptosis regulating genes NLR family apoptosis inhibitory protein (*NAIP,* P=0.033), NLR family pyrin domain containing 1 (*NLRP1,* P=0.037), Peptide transporter 1, (*SLC15A1,* P=0.007) and zonulin (Haptoglobin, *HP*, P=0.010).

Conclusions. In children with IF, current PN was associated with decreased disaccharidase activities and increased inflammation, suggesting that PN requirement negatively affects intestinal adaptation. Duodenal mucosal hyperplasia seemed to have only a limited role in intestinal adaptation after extensive bowel resection in children with SBS and despite weaning off PN, patients showed mucosal inflammation and molecular signs of altered epithelial permeability. Pathologic small bowel dilatation may impair crypt homeostasis through IL-6 signaling and the loss of the ileocecal valve may promote inflammation through increased TLR4 expression. Further studies are necessary to confirm and assess the functional significance of these findings.

TIIVISTELMÄ

Taustatietoa. Vaikka suolen adaptaatiota on tutkittu laajasti eläinkokeissa, tietoa ihmisten suolen adaptaatiosta suolen vajaatoimintaa sairastavilla potilailla on hyvin rajallisesti.

Tutkimuksen tavoite. Tutkia pohjukaissuolen limakalvon homeostaasia suolen vajaatoimintaa sairastavilla lapsipotilailla ja verrokkipotilailla. Suolen vajaatoimintapotilaat olivat joko riippuvaisia suonensisäisestä ravitsemuksesta tai vieroittuneet siitä.

Potilaat ja menetelmät. Kaikkiaan tutkimukseen osallistui 58 suolen vajaatoimintapotilasta iältään 0.5–23-vuotiasta sekä 43 iältään ja sukupuoleltaan vastaavaa verrokkia ilman todettua suolistopatologiaa. Biopsiat kerättiin kliinisesti perusteltujen ohutsuolentähystysten yhteydessä. Potilaiden suonensisäisen ravitsemuksen mediaanikesto oli ensimmäisessä tutkimuksessa (I) 1.2 (IQR 0.6–5.1) vuotta, toisessa (II) 1.4 (0.7–6.5) vuotta ja kolmannessa (III) 0.9 (0.4–2.0) vuotta. Jäljelle jäänyt ohutsuolen pituus oli tutkimuksissa I–III 49 (29–101) cm, 33 (12–60) cm ja 47 (30–60) cm tai vastaavasti 26 (17–48) %, 20 (9–22) % ja 29 (19–43) % odotetusta iänmukaisesta pituudesta. Ileokekaaliläppä oli tallessa (I) 27:llä, (II) kuudella ja (III) 16:sta potilaalla.

Tutkimme pohjukaissuolen disakkaridaasiaktiivisuuksia, tulehdusmuutoksia, limakalvon rakenteellista hyperplasiaa, proliferaatiota, apoptoosia, epiteelin suojaliitosten toimintaa ja ravintoaineiden kuljetusta käyttämällä glukoosioksidaasimenetelmää, histologisia tutkimuksia, immunohistokemiaa ja kvantitatiivista reaaliaikaista polymeraasiketjureaktiota (qPCR) 52 geenille. Ohutsuolen laajenemista tutkittiin suoliston varjoainekuvauksella.

Tulokset. Maltaasin ja sakkaraasin aktiivisuudet olivat suonensisäistä ravitsemusta tarvitsevilla potilailla 1.6 kertaa matalammat ja limakalvojen tulehdusmuutokset yleisemmät (22 % vs. 3 %), verrattuna verrokkipotilaisiin ($P<0.05$ molemmille). Disakkaridaasiaktiivisuudet suonensisäisestä ravitsemuksesta vieroittuneilla potilailla eivät eronneet merkitsevästi verrokkipotilaiden aktiivisuuksista. Seuranta-ajan pituus primäärisuolileikkauksen jälkeen oli suoraan verrannollinen ($r=0.448$ ja $r=0.369$) sekä

jäljellä oleva ohutsuolen pituus kääntäen verrannollinen (r=-0.337 ja r=-0.407) maltaasi- ja sakkaraasiaktiivisuuksiin suonensisäisestä ravitsemuksesta vieroittumisen jälkeen (P<0.05 kaikilla).

Arvioitaessa limakalvon morfologiaa, proliferaatiota ja apoptoosia saatiin toisiinsa verrattavia tuloksia suonensisäistä ravitsemusta tarvitsevilla ja siitä vieroittuneilla potilailla verrattaessa verrokkipotilaisiin. Ohutsuolen iänmukainen jäljellä oleva pituus (%-osuus) oli kääntäen verrannollinen villuksen pituuteen (r=-0.397, P=0.022). Lyhytsuolisyndroomapotilailla havaittiin tilastollisesti korkeammat arvot transformoivan kasvutekijän *(TGF)β2* (potilaat suonensisäisellä ravitsemuksella P=0.035, vieroittuneet potilaat P=0.006) ja Caveolin1:n (*CAV1*) (potilaat suonensisäisellä ravitsemuksella P=0.016, vieroittuneet potilaat P=0.001) lähetti-RNA:n (mRNA) ilmentymisessä verrattuna verrokkipotilaisiin. Lisäksi suonensisäistä ravitsemusta tarvitsevilla potilailla havaittiin korkeammat mRNA määrät verrattuna verrokkipotilaisiin seuraavien geenien kodalla: Claudin 1 (*CLDN1*, P=0.044), Mucin 2 (*MUC2*, P=0.044) ja glukoosin kuljettaja, GLUT1 (*SLC2A1*, P=0.035), sekä matalammat mRNA määrät NLR-perheen CARD 4 geenin (*NLRC4*, P=0.021) kohdalla. Suonensisäisestä ravitsemuksesta vieroittuneilla potilailla löydettiin villusten lamina proprian lisääntynyttä tulehdusta (P=0.033) sekä kohonnutta tuumorinekroositekijägeenin (*TNF*, P=0.027) ja *TGF-β2*:n (P=0.006) mRNA ilmentymää verrattaessa verrokkipotilaisiin. Patologinen ohutsuolen laajentuminen liittyi tutkimuksessamme matalimpiin kryptiin (P=0.045) ja alhaisempaan interleukiini (*IL)-6*:n mRNA ilmentymään (P=0.008), kun taas suolen laajentuminen korreloi negatiivisesti *IL6*:n määrään (r=-0.609, P=0.004) ja seuraaviin proliferaatiomarkkerigeeneihin: Proliferatiivinen soluydinantigeeni (*PCNA*, r=-0.439, P=0.046) ja proliferaatiomarkkeri Ki-67 (*MKI67*, r=-0.625, P=0.002). Ileokekaaliläpän menetys liittyi korkeimpiin mRNA ilmentymiin Tollin kaltainen reseptori 4-geenin (*TLR4,* P=0.037), *TGFβ1*:n (P=0.043) ja *CAV1*:n (P=0.025) sekä apoptoosia säätelevien geenien NLR-perheen apoptoosia inhiboiva proteiinin (*NAIP,* P=0.033), NLR-perheen pyriinidomeeni 1:n (*NLRP1*, P=0.037), Peptidi-transportteri 1:n, (*SLC15A1*, P=0.007), ja zonuliinin (Haptoglobiini, *HP*, P=0.010) kohdalla.

Johtopäätökset. Suolen vajaatoimintaa sairastavilla lapsilla riippuvuus suonensisäisestä ravitsemuksesta liittyi vähentyneeseen disakkaridaasiaktiivisuuteen ja lisääntyneeseen

tulehdukseen, mikä viittaa siihen, että suonensisäinen ravitsemus vaikuttaa negatiivisesti suoliston adaptaatioon. Pohjukaissuolen limakalvon hyperplasialla näytti olevan vain rajallinen rooli suoliston adaptaatiossa lyhytsuolisyndroomaa sairastavilla lapsilla merkittävän suoliresektion jälkeen, ja suonensisäisestä ravitsemuksesta vieroittumisesta huolimatta potilailla havaittiin lisääntyneitä pohjukaissuolen limakalvon tulehdusmuutoksia ja merkkejä permeabiliteetin muutoksista. Patologinen ohutsuolen laajentuminen saattaa heikentää kryptien homeostaasia IL-6-signaalireitin kautta ja ileokekaaliläpän menetys saattaa lisätä tulehdusmuutoksia lisääntyneeseen *TLR4*-ilmentymään liittyen. Lisätutkimukset ovat välttämättömiä tulevaisuudessa, jotta voimme varmistua näistä tuloksista sekä arvioida niiden toiminnallista merkitystä.

TABLE OF CONTENTS

ORIGINAL PUBLICATIONS

This thesis is based on the three following publications:

I Sanaksenaho G, Mutanen A, Koivusalo AI, Merras-Salmio L, Pakarinen MP. Duodenal Disaccharidase Activities During and After Weaning off Parenteral Nutrition in Pediatric Intestinal Failure. *J Pediatr Gastroenterol Nutr.* 2017 May;64(5):777-782.

II Sanaksenaho G, Mutanen A, Godbole N, Kyrönlahti A, Koivusalo A, Lohi J, Pihlajoki M, Heikinheimo M, Pakarinen MP. Parenteral Nutrition Dependent Children with Short Bowel Syndrome Lack Duodenal Adaptive Hyperplasia but Show Molecular Signs of Altered Mucosal Function. *JPEN.* 2020 Jan;44(7):1291-1300.

III Sanaksenaho G*, Mutanen A*, Godbole N, Merras-Salmio L, Hukkinen M, Kivisaari R, Kyrönlahti A, Pihlajoki M, Lohi J, Heikinheimo M, Pakarinen MP. Compromised Duodenal Mucosal Integrity in Children with Short Bowel Syndrome After Adaptation to Enteral Autonomy. *J. Pediatr.Surg*. 2020 Oct;S0022-3468(20)30714-4.

*equal contribution

The publications are referred to in the text by their roman numerals (I-III) with the permission of the original publishers. Any information which has not been published previously is mentioned in the text.

ABBREVIATIONS

ABCG5	ATP Binding Cassette Subfamily G Member 5 (sterol transporter)
ABCG8	ATP Binding Cassette Subfamily G Member 8 (sterol transporter)
ACTB	Beta-actin (β-actin)
AIR	Autologous Intestinal Reconstruction
ATP	Adenosine Triphosphate
BAX	B-Cell lymphoma (BCL2) Associated X
BCL2	B-Cell lymphoma 2
B2M	β-2-microglobulin
CAR	Coxsackie and Adenovirus Receptor
CARD	Caspase Recruitment Domain
CASP1	Caspase 1
CASP4	Caspase 4
CAV1	Caveolin 1
CDH1	Cadherin 1
CLDN1	Claudin 1
CLDN2	Claudin 2
CLDN3	Claudin 3
CVC	Central Venous Catheter
DNA	Deoxyribonucleic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELBW	Extremely Low Birth Weight
EN	Enteral Nutrition
F11R	Junctional Adhesion Molecule 1
FABP2	Fatty Acid Binding Protein
FATP4	Long-chain Fatty Acid Transport Protein 4
FGF7	Fibroblast Growth Factor 7
FIG	Figure
GCG	Glucagon
GLP-2	Glukagon Like Peptide-2
GLP2R	Glucagon Like Peptide 2 Receptor
GLUT1	Glucose Transporter 1
H&E	Hematoxylin and Eosin
HD	Hirschsprung's Disease
HP	Haptoglobin, Zonulin
HPR	Haptoglobin-related Protein
HPRT1	Hypoxanthine guanine phosphoribosyl transferase I
HSP90AB1	Heat shock protein 90kDa α (cytosolic), class B member 1
ICV	Ileocecal Valve
IELC	Intraepithelial Leukocytes Count

IF	Intestinal Failure
IFALD	Intestinal Failure Associated Liver Disease
IFNG	Interferon-gamma
IL1A	Interleukin 1α
IL1B	Interleukin 1β
IL6	Interleukin 6
IL8	Interleukin 8
IL10	Interleukin 10
IL17A	Interleukin 17α
IL18	Interleukin 18
IQR	Interquartile Range
JAM	Junctional Adhesion Molecule
LILT	Longitudinal Intestinal Lengthening and Tailoring
MKI67	Marker of Proliferation Ki-67
MMCs	Migrating Myoelectric Complexes
mRNA	Messenger Ribonucleic Acid
MUC2	Mucin 2
NAIP	NLR family Apoptosis Inhibitory Protein
NEC	Necrotizing Enterocolitis
NLR	Nod- Like Receptor
NLRC4	NLR Family CARD Domain Containing 4
NLRP1	NLR Family Pyrin Domain Containing 1
NLRP3	NLR Family Pyrin Domain Containing 3
NLRP6	NLR Family Pyrin Domain Containing 6
NPC1L1	Niemann-Pick C1-Like 1 (sterol transporter)
OCLN	Occludin
PCNA	Proliferating cell nuclear antigen
PEPT1	Peptide Transporter 1
PIPO	Pediatric Intestinal Pseudo Obstruction
PN	Parenteral Nutrition
qPCR	quantitative Polymerase Chain Reaction
rhGH	Growth Hormone
RNA	Ribonucleic Acid
RPLP0	Ribosomal protein, large, P0
SBA	Small Bowel Atresia
SBD	Small Bowel Diameter
SBDR	Small Bowel Diameter Ration
SBS	Short Bowel Syndrome
SCFAs	Short Chain Fatty Acids
SLC	Solute Carriers
SLC15A1	Solute Carrier Family 15 (oligopeptide transporter)
SLC27A4	Solute Carrier Family 27 (fatty acid transporter) Member 4
SLC2A1	Solute Carrier Family 2 (facilitated glucose transporter) Member 1

SLC5A1	Solute Carrier Family 5 (sodium/glucose cotransporter) Member 1
SLGT1	Sodium/Glucose Cotransporter 1
SMA	Superior Mesenteric Artery
STEP	Serial Transverse Enteroplasty
TGFB1	Transforming Growth Factor β1
TGFB2	Transforming Growth Factor β2
TLR2	Toll-like Receptor 2
TLR3	Toll-like Receptor 3
TLR4	Toll-like Receptor 4
TLR5	Toll-like Receptor 5
TLR8	Toll-like Receptor 8
TLR9	Toll-like Receptor 9
TNF	Tumor Necrosis Factor
TPN	Total Parenteral Nutrition
VLBW	Very Low Birth Weight
ZGLP1	Glucagon Like Peptide 1
ZO	Zonula Occludens

INTRODUCTION

The condition where the intestinal ability to manage the nutrient, electrolytes, and fluids absorption is not enough to ensure a child's needs for normal growth is called pediatric intestinal failure (IF) [1]. Most of the cases are caused by short bowel syndrome (SBS) where surgical removal of the small bowel has led to malnutrition and the need for parenteral nutrition (PN) [2]. In the pediatric population, the reasons for SBS are mainly necrotizing enterocolitis (NEC) or congenital anomalies such as small bowel intestinal atresia (SBA), malrotation, gastroschisis, and long segment Hirschsprung`s disease [3,4]. SBS is a rare and severe condition [5]. The risk of developing SBS increases significantly in premature babies [6]. Initial surgical treatment aims to preserve the maximal potential length of the intestine and to restore bowel continuity as soon as possible in case of endostomy [7]. After major resection, the remaining intestine undergoes adaptation and meanwhile, PN is necessary to sustain life, to enhance energy balance and to prevent nutritional deficiencies in children [1,8]. However, PN used after bowel resection also predisposes to severe infections, intestinal failure associated liver disease (IFALD) and metabolic complications [9]. Enteral autonomy is considered one of the main goals in the treatment of children with IF [1,5].

Many patients are able to wean off PN due to intestinal adaptation, which is a complex and multifactorial process [3,5,10,11]. In intestinal adaptation, the remaining intestine increases the absorptive area and capacity by villous hyperplasia, crypt elongation, increased activity of digestive enzymes, bowel lengthening, and an increase in mass and dilatation [5,11-13]. Several humoral factors participate in this process by modifying hormone release, transit time, bowel contractions, intestinal epithelial cell production, proliferation, migration and apoptosis [7,11,14,15]. The important anatomical details for the function of the remaining bowel are small bowel length and dilation, the presence of the ileocecal valve (ICV), the length of the remaining colon and the location of the possible endostomy [1,10,16,17]. Most of the adaptive changes occur in the small intestine and are actively studied in animal models [14,18,19]. Despite this, data on duodenal adaptive changes remained limited [12,15,20].

The focus of the present study was on the duodenal adaptation in children with SBS during and after weaning off PN compared to control individuals without known gastrointestinal disease. Our research group assessed duodenal disaccharidase activities and signs of inflammation (I) based on pathological reports. Furthermore, duodenal biopsies were evaluated considering the mucosal hyperplasia, inflammation, barrier function and nutrient transport by using histology, immunohistochemistry and qPCR for selected key genes, both for patients currently on PN (II) and after achieving total enteral autonomy (III). Analysis was done to see how the remaining bowel anatomy and time after bowel resection and PN affected the results. The impact of excessive dilatation of the remaining small bowel was studied in weaned-off SBS patients (III).

In this study, our research group had two main hypotheses. Firstly, the dependence on PN is associated with decreased duodenal disaccharidase activities and mucosal inflammation in children with IF and SBS (I, II). Secondly, that the hyperplastic mucosal adaptive response and molecular signs of enhanced mucosal proliferation, apoptosis, inflammation, and permeability would be seen in children with SBS (II, III).

REVIEW OF THE LITERATURE

1 Embryology

The gastrointestinal tract is visualized when the embryo is 14 days old [21]. During the subsequent days, the duodenojejunal and cecocolic loops are formed. Both loops gradually elongate, move and undergo specific rotation within ten weeks, while the small and large intestines achieve their final positions. [21,22] This process is described in more detail in the chapter 'Malrotation'. The length of the small bowel is about 70 cm at 24 weeks of gestational age and doubles during the final months of pregnancy, reaching a length of approximately 155 cm by the due date [23]. The length of the colon grows between 24 and 40 gestational weeks from a mean of 22 to 32 cm [23].

2 Normal Bowel Anatomy and Function

2.1 Small intestine

The remaining intestinal anatomy is strongly associated with the outcomes in SBS children [3,4,24]. Rapid growth of the small bowel continues after birth [23,25]. While the mean small bowel length in babies at the age of six months is about 240 cm, at five years it is about 420 cm [23]. It then reaches its full length during adolescence of up to 560 cm (with substantial variation in adults between 360-1090 cm) [25]. Major small bowel resection most consistently leaves the duodenum as the part of the remaining intestine, despite this, little is known about duodenal adaptation while the adaptation of the jejunum and ileum have been extensively researched [11,26]. Most of the vitamin and nutrient absorption takes place in the duodenum and jejunum, while the ileum deals with the utilization of fats bounded to bile acids, vitamin B12, fat-soluble vitamins, fluids, and electrolytes [27].

2.1.1 Duodenum

The first part of the small intestine is the duodenum (Fig 1); it has very high rate of contractions showing rapid propagation velocity [mean 28 (± 20) cm s^{-1}] [28]. It is the

shortest, widest, and most fixed part of the small bowel with no mesentery, divided into four parts (superior, descending, horizontal and ascending, Fig 1.) [29]. The bile duct descends to the second (II) part of the duodenum (Fig 1). It secretes bile which is formed by hepatocytes and stored in the gallbladder from which it is released between meals [30,31]. Bile acids are necessary for the absorption of triglycerides, cholesterol and lipid-soluble vitamins [30]. The pancreatic duct joins with the bile duct before their common opening in the posterolateral wall of the descending duodenum called *papilla Vateri* (Fig 1.) [32]. Pancreatic juice contains a great amount of proteins required for proteolysis [33]. Duodenal mucosa harbors many small peptide and amino acid sensors (for instance solute carriers and oligopeptide transporters) and several receptors to recognize carbohydrates and fatty acids. The activation of several chemosensors leads to the secretion of gastrointestinal hormones [34].

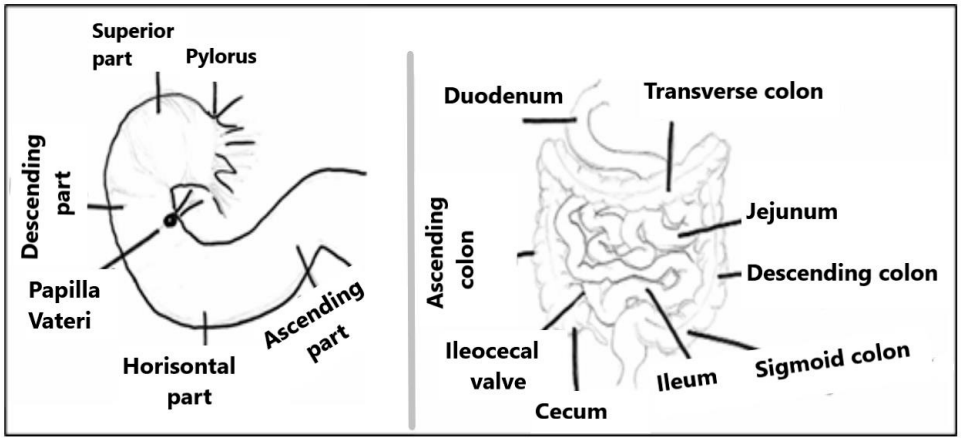
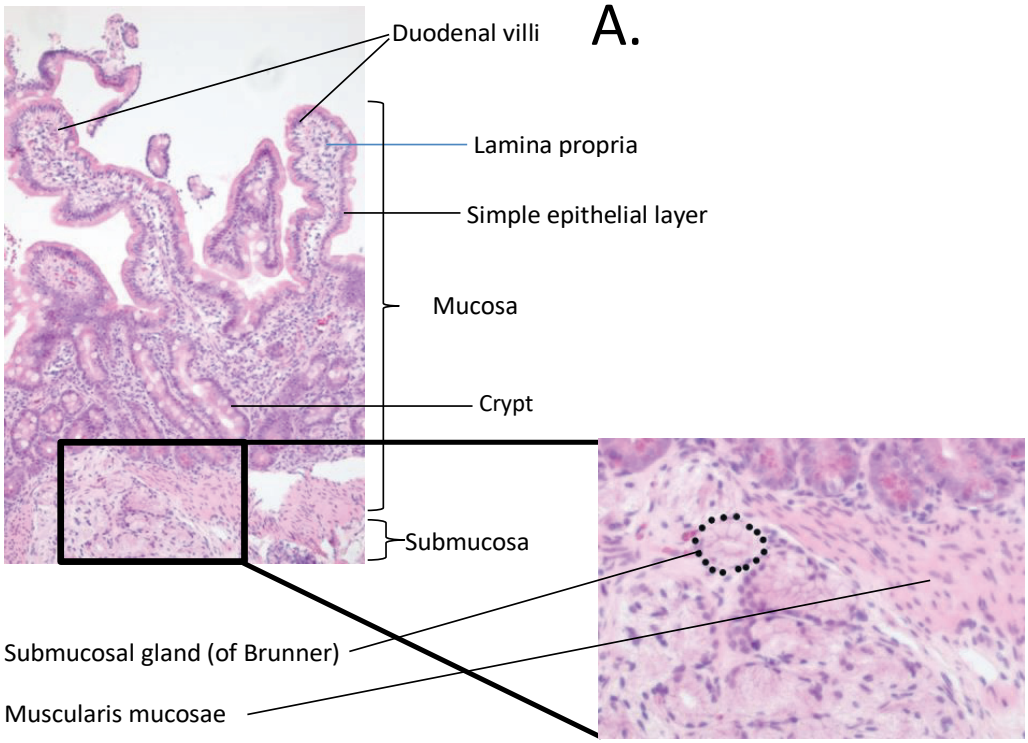
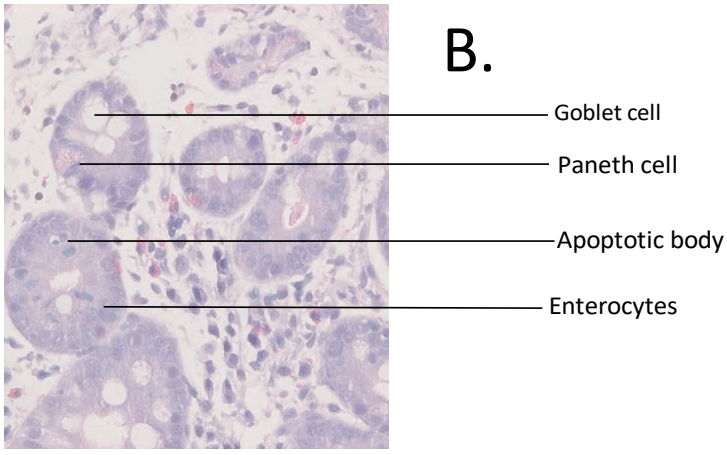


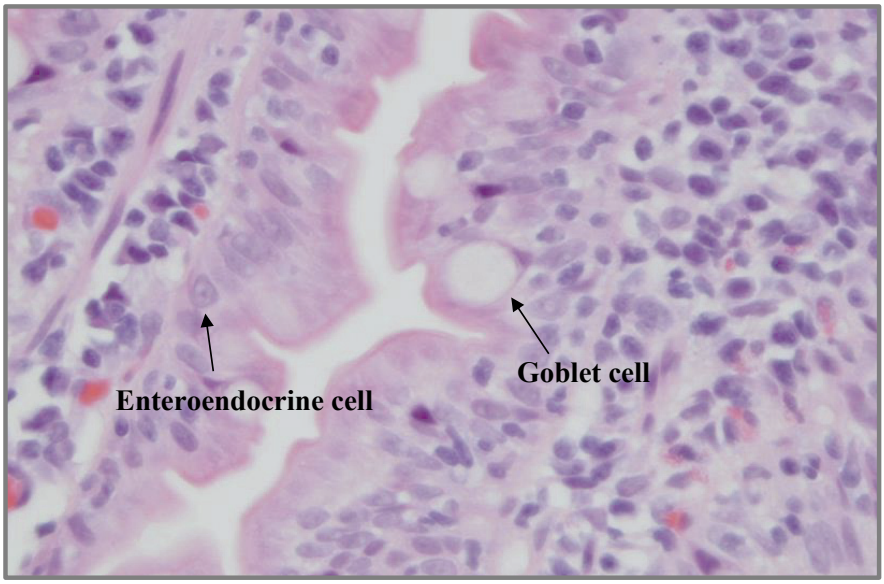
Figure 1. Anatomy of small and large intestine.

Histological structures of duodenal mucosa are shown in Figure 2. The mucosa consists of crypt-villus units and a muscular layer, while submucosa harbor submucosal glands called Brunner`s glands (Fig 2). Brunner`s glands are able to secrete alkaline and fluid which together neutralize the gastric acid of partly digested food coming from the stomach [35]. Small intestinal mucosa represents normally low-grade, physiologic inflammation due to continued exposure to luminal bacteria, mitogens and many Toll-like receptor (TLR) ligands [36]. The epithelial layer harbors approximately one T cell

per ten epithelial cells and is covered by a mucus, gel-like protective layer [36,37]. Epithelial cells are responsible for the absorptive function, Goblet cells for the production of mucin, enteroendocrine cells for the hormone secretion and Paneth cells of the crypts for the secretion of antibacterial peptides, digestive enzymes and growth factor (Fig 2 B, C) [38,39]. One third of all cells of the villous lamina propria in the small intestine are T cells, and they participate in immune response. Dendritic cells and macrophages of lamina propria may have pro- and anti-inflammatory roles in the development of intestinal inflammation. [36] On the apical side of the intracellular space lies the structures of the permeability barrier called tight junctions. These protein complexes cooperate with each other and other intracellular proteins in a very intricate way. Tight junctions are not exactly stable complexes, they are able to adapt to changing environment by adjusting regulatory pathways. [39] They prevent bacteria from entering the lamina propria [36,40]. Sub-adjacent to the tight junctions are adherens junctions responsible for the cell recognition, which also mediate intercellular associations [39]. Desmosomes lie under the adherens junctions and Cadherin tails connect with the intermediate filaments and plakins [41]. An illustration of the main proteins and network between tight and adherens junctions and desmosomes is shown in Figure 3.


A.
Duodenal villi
Lamina propria
Simple epithelial layer
Mucosa
Crypt
Submucosa
Submucosal gland (of Brunner)
Muscularis mucosae


B.
Goblet cell
Paneth cell
Apoptotic body
Enterocytes



C.

Figure 2. A. Duodenal histology, structures of mucosa and submucosa. B. Cross sectional view of the crypts. C. Enteroendocrine and Goblet cells on villi.

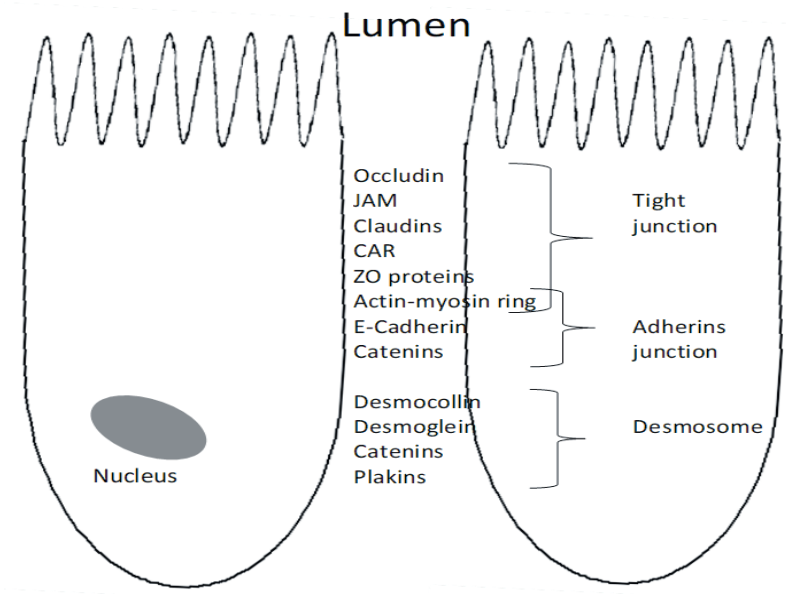


Figure 3. The main proteins forming instestinal barrier function. Network between tight and adherens junctions and desmosomes. CAR, coxsackie and adenovirus receptor; JAM, junctional adhesion molecule; ZO, zonula occludens.

2.1.2 Jejunum and ileum

The jejunum begins from the duodenojejunal junction and joins the ileum, which ends at the ileocecal junction (Figure 1). The jejunum takes for the proximal two-fifths, while ileum comes to three-fifth of the distal small bowel [29]. Food propagates in the jejunum much slower than in the duodenum with a slightly, but significantly, higher postprandial velocity in the jejunum than in the ileum [1.6 ± 0.1 vs. 1.4 ± 0.1 cm s^{-1}, $P < 0.05$] [42]. The jejunum is responsible for the utilization of several nutrients and the ileum maintains mostly the fluid balance [27].

2.2 Large intestine

After birth, the large intestine grows extensively during the first six months of life, achieving a length of 122 cm at the age of five [23]. This is approximately the full length of the large bowel, as Saunders and colleagues reported a mean range for the adult population of 114 cm (68-159) [43]. The large intestine includes the appendix, caecum, ascending, transverse, descending, and sigmoid colon (Figure 1). The ileocecal valve controls the release of intestinal contents into the colon and acts as a gateway preventing reflux between the ileum and large intestine [44].

3 Short Bowel Syndrome in children

3.1 Definition, incidence, and mortality

Pediatric short bowel syndrome (SBS) is a rare and severe condition in which the mass of a well-functioning intestine is not enough for adequate digestion and absorption of nutrients as well as the fluid requirements to maintain normal growth in children [2,5]. Several definitions are used for SBS either regarding the remaining small bowel length (<25 or <50% of expected) or dependency and duration of PN (42-90 days) (Table 1) [3,4,6,45-48]. SBS may also be classified into three subtypes based on the remaining bowel anatomy and surgical treatment. The first group includes small bowel resection with a small bowel anastomosis and intact colon, mostly with some preserved ileum. The second group consists of small bowel and partial colon resections with small bowel-colon anastomosis. The third group includes patients with extensive small bowel resection and a high-output jejunostomy [24].

Wales *et al*. from Canada reported a population-based incidence of pediatric SBS of 24.5 per 100 000 live births, being significantly higher in premature babies (<37 gestational age 353.7 vs. term babies 3.5 per 100 000] [6]. Prematurity alone is associated with a higher risk for SBS, and very or extremely low birth weight neonates have a tremendously increased risk for the incidence of surgical necrotizing enterocolitis (NEC) and SBS [45,46].

The comparison of mortality rates between studies is complex as there is no clear standardly used definition for the SBS and the follow-up period. Patient age and the remaining bowel anatomy vary between studies. [3,4,6,24,45-48] In addition, patients who experience early death are usually excluded from the studies [6,24,47] (Table 1). The most common diagnoses and their distribution in children with IF or SBS are shown in Table 2 [3,4,6,45-48]. The studies described in Tables 1 and 2 represent single- and multi-center studies with IF and/ or SBS pediatric patients of different ages from Europe, USA and Canada [3,4,6,45-48].

Table 1. Definitions of IF and SBS, incidence and fatality rate in different studies.

	P.Wales *et al.* ***J Pediatr Surg. 2004***	**Cole *et al.*** ***Pediatrics. 2008***	**Salvia *et al.*** ***J Pediatr. 2008***	**Fullerton *et al.*** ***J Pediatr Surg. 2016***	**Merras-Salmio *et al.*** ***JPEN 2018***	**Thompson *et al.*** ***Annals of Surgery 1995***	**Squires *et al.*** ***J Pediatr. 2012***
Study period and follow up	1997-1999 (follow up mean 2 years)	2002-2005 (follow up for ELBW 2 years, for VLBW 1 year)	2003-2004 (follow up median 3 years)	2002-2014 (follow-up median 5 years)	1984-2017 (follow up median 7 years)	1980-1994 (follow up mean 6 years)	2000-2005 (follow-up median 2 years)
Country	Canada	USA	Italy multi-center	USA	Finland	USA	Canada, USA multi-center
Number of patients w/ SBS and/or IF	40	89	16 /26	313 (IF)	78 /100	112	272 (IF)
Patients and/or definition of SBS	TPN >42 days post-resectionally or SB length <25% of expected	VLBW, ELBW. Intestinal resection, which led to the need for PN	TPN > 4 weeks or the need of partial PN> 3 months/ PN >42 days or SB length <25% of expected	PN support >90 days	PN >60 days or SB length <50% of expected	<16 years and SB< 120 cm and/or PN on discharge	Age <12 months and PN 60 out of 74 consecutive days
Exclusion criteria (Excluded N)	Neonates with pyloric stenosis, life-threatening anomalies, chromosomal abnormalities, comorbid conditions. (N=44)	Admission to the hospital after 14 days of birth and death within 12 hours after delivery.	NR	NR	Patients with underlying malignant disease.	Death in the hospital after massive bowel resection.	NR
Incidence	24.5/100000 (per life births)	7/1000 (among all VLBW babies n=12 316)	1/1077 (per life births)	NR	NR	NR	NR
Mortality rate (%)	38	31 (ELBW) 18 (VLBW)	NR	6	8 (1984-2011) 0 (2011-2017)	13	25

ELBW, extremely low birth weight; IF, intestinal failure; SB, small bowel; NR, not reported; PN, parenteral nutrition; SBS, short bowel syndrome; TPN, total parenteral nutrition; VLBW, very low birth weight.

Table 2. Etiology of IF in previously reported studies.

	Wales *et al.* n=40 SBS	**Cole *et al*. n=89 SBS**	**Salvia *et al.* n=26 IF/ n=16 SBS**	**Fullerton *et al.* n=313 IF**	**Merras-Salmio *et al.* n=78 SBS**	**Thompson *et al.* n=112 SBS**	**Squires *et al*. n=272 IF**
Patient age	Neonates' mean GA 37 w	VLBW (71% at GA 25-28 w, 14% < 25 w and 14% ≥29 w)	Neonates median GA 32 w	<18 y	median follow-up age 6.4 y (IQR 3.1-11)	<16 y	<1 y
Diagnosis (%)							
Necrotizing enterocolitis	35	96	31/38	30	45	33	26
Gastroschisis/AWD	13	NR	8/6	23	5	NR	16
Intestinal atresia	10	NR	35/44	17	12	21	10
Volvulus	10	2	0	11	18	32	9
Long segment Hirschsprung's disease and/or PIPO	3	0	12/6	12	13*	NR	4
Other	30	2**	15/6	7	8**	14	35***

AWD, abdominal wall defect; PIPO, pediatric intestinal pseudo-obstruction; ELBW, Extremely low birth weight; GA, gestational age (weeks); IF, intestinal failure; IQR, interquartile range; NR, not reported; PN, parenteral nutrition; SB, small bowel; SBS, short bowel syndrome; TPN, total parenteral nutrition; VLBW, very low birth weight, w, week; y, years.

* Patients with Hirschsprung's disease and >50 % small bowel resection. ** Patients with gastroschisis or intestinal atresia or both. *** 7% with other single diagnosis and 28% with multiple single diagnoses.

3.2 Etiology

3.2.1 Necrotizing enterocolitis

Necrotizing enterocolitis (NEC) is the most common etiology of SBS (Table 2), characterized by inflammation, ischemia and infection leading to bowel necrosis, with or without perforation [49]. The prevalence of NEC is approximately two percent, being a rare condition in full-term neonates and incidence grows tremendously with decreased birth weight and prematurity [among term neonates with birth weight >2000 g 0.1- 0.4% vs. among preterms with very low birth weight (VLBW) or extremely low birth weight (ELBW) infants 8-14%] [45,50-52]. NEC does not always lead to the intestinal resection as less severe cases can be treated medically, and in the case of a limited affected area, the resection does not lead to SBS [49]. The need for surgical intervention in full-term infants varies between 31% and 40%, and in pre-terms between 47% and 58% [45,50,51,53]. A very accurate study on low birth weight infants showed that surgery was performed in 50% of VLBW infants and 62% of ELBW infants, leading to SBS in 18% and 16% of cases, respectively [45].

3.2.2 Gastroschisis

Gastroschisis is a congenital anomaly of the anterior abdominal wall, where the intestine herniates outside, mostly located to the right of the umbilicus. It is associated with prematurity and patients tend to suffer from the initial, inherent gut dysmotility which requires at least temporary management with PN. [54] The global incidence of gastroschisis has been increasing but the reasons are unknown [55]. The treatment considers either primary closure or a silo, transparent silastic bag equipped with a spring loaded ring [54-56].

Complex gastroschisis is complicated by atresia, necrosis, volvulus or perforation and the need for further intestinal resection. It occurs in approximately 20% of patients with gastroschisis (17-21%). [54,57,58] The condition is associated with an increased risk for longer mechanical ventilation, a longer need for PN, for developing IF, increased incidence of NEC and sepsis, and significantly higher mortality [57,58]. Mutanen *et al*. reported a 98% incidence (eight out of nine) of developing IF among patients with

complex gastroschisis, while respective incidence in patients with isolated gastroschisis was nine percent (3 out of 34) [57].

3.2.3 Malrotation

The development of the intestinal tract during the first weeks of pregnancy (4 to 10 weeks) and manifestations of patients with volvulus are well described in a study by Snyder and Chaffin in 1954. In a five mm embryo, intestines are in the form of a straight tube, the duodenojejunal loop being in the midline above the superior mesenteric artery (SMA). During the following weeks, it moves and rotates up to 270° in a counterclockwise direction. At the same time, the cecocolic loop, which includes the ileum, cecum, and colon, moves beneath and to the left of the SMA, then drops into the abdomen. It continues rotating superiorly and anteriorly regarding the SMA, achieving a final curve of 270° in a counterclockwise direction. [22]

Disruptions during the rotation and fixation of the duodenojejunal and cecocolic loops result in malrotation due to failure of the mesentery to attach, affecting especially the duodenum and ileum [22]. This predisposes to rotation around the axis of the SMA, eventually leading to obstruction of the intestinal lumen, vein drainage, and arterial supply [59]. Malrotation with midgut volvulus leads to death if the intestine is extensively ischemic and sepsis occurs. The Ladd procedure is performed in the surgery in which the detorsion of the volvulus is performed in a counterclockwise direction. The small intestine is then placed on the right while colon on the left side of the patient. Appendicectomy is also usually performed [60].

3.2.4 Small bowel atresia

Small bowel atresia (SBA) is a congenital anomaly with a wide spectrum of manifestations [61]. The pathophysiology has been described to relate, at least partly, to vascular accidents which result in local ischemia, aseptic necrosis and finally, either stenosis or atresia [62].

Different types of SBA have been described. There can be an intraluminal membrane with a muscular coat continuity between proximal and distal parts (Type I), or both intestinal segments could be blind with a cord-like segment between (II), or intestinal

segments could be separated due to mesenteric defect (Type III a). Types I-IIIa are leading diagnoses among patients with SBA. Apple peel atresia (Type III b) results from an extensive infarction of the midgut, leading to proximal jejunal atresia, a poorly developed and shortened small bowel with chronic ischemic mucosal and muscular layer damage. Multiple atresias (Type IV) are mostly located in the jejunum and ileum but can also be situated in the colon; the condition is often associated with a shortened small bowel. [61]

SBA can lead to significant bowel resection and SBS. Casaccia *et al*. reported a study of 44 neonates with congenital anomalies diagnosed and treated between 1998 and 2003 in Italy. Twenty-three of the neonates had jejunoileal atresia and SBS developed in nine of them (39%). Among them, SBS occurred in all patients with Apple peel atresia, in 75% with multiple atresia, and in 8% with Type I-IIIa atresia. Other etiologies for SBS in this group were meconium peritonitis and fetal intestinal volvulus (n=10). SBS led to a significant increase in the number of septic episodes, delays in motor development, and the length of the hospital stay. Four patients died during follow-up, three of them with SBS, but the study lacked information about their diagnoses. [63]

3.2.5 Extended aganglionosis of Hirschsprung's disease

Hirschsprung's disease (HD) is characterized by a lack of ganglion cells in the myenteric submucosal plexuses of the distal bowel followed by intestinal obstruction and an absence of peristalsis, which leads to the dysfunctional bowel segment. Ganglion cells' differentiation, maturation, and migration to the distal intestine occur within the first 13 gestational weeks. The arrest during this process leads to aganglionotic bowel, most commonly at the rectum or rectosigmoid level (80% of patients). [64,65] In the remaining patients, the aganglionosis extends beyond the rectosigmoid, involving the descending colon, transverse colon or may involve the entire colon with a short segment of the terminal ileum [65]. In extensive HD (<1% of patients), the entire, or almost the entire, intestine is aganglionotic from the proximal small bowel to the rectum leading to SBS with significant morbidity [65,66].

3.2.6 Pediatric intestinal pseudo-obstruction and Congenital enteropathies

Pediatric intestinal pseudo-obstruction (PIPO) refers to a heterogenous group of rare and severe primary and secondary disorders characterized by symptoms of bowel obstruction and abnormal intestinal contractility which leads to IF. Based on histopathology, contractility patterns and genetics, PIPO may be classified as myopathy, neuropathy, and mesenchymopathy. [65,67,68] Congenital enteropathies are rare congenital conditions such as microvillus inclusion disease, intestinal epithelial dysplasia (tufting enteropathy), enteroendocrine cell dysgenesis, and autoimmune enteropathy, presenting with early-onset severe watery diarrhea and leading to IF [65,69]. The enteropathies are characterized by major disruption in intestinal histology, including villous atrophy, abnormal crypts or brush border, abnormalities in Paneth cell or enterocytes, or a lack of endocrine cells [69].

3.2.7 Other causes of short bowel syndrome

In children, other more rare causes of SBS are trauma, tumors, arterial and venous thrombosis, complicated intussusception, and inflammatory bowel disease [2,6,24,63,65].

3.3 Parenteral nutrition

Parenteral nutrition (PN) was introduced in the 1960s for patients with IF with the aim of sustaining life and providing growth in children [70]. Since then, PN treatment has undergone colossal developments in safety and effectiveness, and is possible to perform at home, which shortens hospitalization time and improves quality of life [70-73]. PN supplies energy, fluids, electrolytes, micronutrients, and vitamins. As a result, each child has a personalized plan for PN consistency and implementation [70,71]. Despite the progress in safety aspects, dependence on PN may lead to several serious complications which are described later in chapters 3.4.1 – Intestinal failure-associated liver disease and 3.4.3 – Sepsis [73,74].

3.4 Outcomes and complications in short bowel syndrome

A few decades ago, most infants died after major small bowel resection [75]. However, due to improvements in surgical, nutritional, and central line management, and a

multidisciplinary approach, most of these children can now survive and achieve full enteral autonomy [3,10,24]. In a study from Finland with 100 pediatric IF patients studied between 1984 and 2017, eight died during follow up and no deaths were reported after 2011 [3]. In addition to that, two of the 12 children with very short bowel syndrome (remaining small bowel length 25 cm or less) treated in Finland between 1988 and 2013 died due to liver failure and thus, the overall survival rate for SBS patients with very short bowel was 83% [26]. Premature babies with SBS and low birth weight have been reported to show high fatality rates, but most survivors were able to achieve enteral autonomy [45]. Patient age, prematurity, PN dependency, septic episodes, and the remaining bowel anatomy affect the long-term outcomes and risk of complications in pediatric SBS [65]. Despite improved survival rates, patients with SBS are at risk of PN-related complications, liver disease, septic episodes, central line-related complications, intestinal bacterial overgrowth, impaired renal function, and bone disease [6,9,24,45,63].

3.4.1 Intestinal failure-associated liver disease

During intestinal adaptation, SBS patients need PN support to sustain life but long-term PN is closely associated with intestinal failure-associated liver disease (IFALD), affecting especially neonates [9,76]. Kurvinen *et al*. reported relatively high (25%) incidence of IFALD in neonates with IF one month after weaning off PN [76]. Recent studies have showed that dextrose and plant sterols, components of PN, may be linked with PN-associated cholestasis in premature neonates and pediatric IF patients [77,78]. IF patients on current PN in previous study demonstrated liver inflammation and cholestasis more often than weaned off patients, accompanied with higher RNA expression of pro-inflammatory cytokines [interleukin 6 (*IL6*) and tumor necrosis factor (*TNF*)] [78]. The pathogenesis is thought to begin when components of PN and small bowel resection lead to small bowel bacterial overgrowth, intestinal dysbiosis, and increased intestinal permeability [78,79]. Inflamed intestinal mucosa thus absorbs lipopolysaccharides into the portal circulation, affecting the hepatocytes directly or activating hepatic macrophages through the TLR-4 binding [79]. Macrophages release proinflammatory cytokines which causes suppression of the bile salt export and reduced canalicular secretion of conjugated bile acids, bilirubin and cholestasis [78].

3.4.2 Small intestinal bacterial overgrowth

Small intestinal bacterial overgrowth (SIBO) is a condition of the small bowel with the presence of excessive bacteria [2]. It is associated with impaired intestinal motility and bowel dilatation. This is followed by the stasis of unabsorbed nutrients, which promotes the excessive growth of bacteria. [80] ICV slows transit time from the small to large bowel and acts as a gateway which prevents bacterial colonization of the small intestine under normal physiological circumstances. The absence of ICV predisposes to the transition of colonic bacteria to the small bowel and increases the risk of bacterial overgrowth. [81] SIBO along with the presence of cholestasis has been reported to be associated with the absence of ICV and gram negative bacteria overgrowth [2]. Intestinal microbiota has been studied in 23 pediatric IF patients (of whom 13 had SBS) and compared to the samples of 58 control individuals. The study showed more primitive microbiota in both diversity and richness in IF patients. They demonstrated, inter alia, an 18-fold average increase of bacilli (mostly lactobacilli), a 10-fold increase of proteobacteria, and a 5-fold increase of actinobacteria, while *Clostridium Clusters IV* and *XIVa* were lacking, which are usually present in a healthy intestine. Overabundance of lactobacilli has been reported among SBS patients with liver injuries after weaning off PN. Proteobacteria, instead, was connected to liver injury, prolonged PN-delivery and inflammation of the liver and intestine. [82] Lactobacilli has been shown to dominate in patients with the intact ICV (n=2) and proteobacteria in patients without ICV (n=7) in a small series study of SBS patients [20]. In a study of 49 SBS children, where all seven children with prolonged PN dependency and only half of patients (n=23) who were eventually weaned off PN, showed bacterial overgrowth [80]. Overall, the microbiota of patients with IF and SBS seems to differ from the microbiota of healthy children and may affect intestinal adaptation.

3.4.3 Sepsis

Sepsis is one of the major causes of death in SBS patients [3,6,45,83]. The administration of PN through the central line is essential for patients but increases the risk of catheter related bloodstream infections [9]. The use of the ethanol lock prophylaxis bundle notably reduced the risk of central line catheter bloodstream infections in IF pediatric patients and has been successfully performed at both hospitals and homes. Thus, the incidence in these

patients was reported to be 0.42-0.63 per 1000 catheter days. [3,73] Neonates with IF seem to be at a higher risk for septic infections than older children during PN-delivery. A previous report showed neonates also having a higher incidence of *Staphylococcus aureus* as a main pathogen when compared to older children (56% vs 33%) [76].

The origin of non-central line septic infections has been reported to relate to the intestinal or respiratory system [16,84]. After small bowel resection, the remaining small bowel dilates and together with disturbed motility may lead to SIBO [80]. Children with SBS and a pathological small bowel dilatation ratio (small bowel diameter/ height of the fifth lumbar vertebra >2.17) on PN have been shown to experience intestinal derived bloodstream infections 15 times more frequently than those with a normal small bowel diameter ratio. In children with IF, PN dependency and small bowel dilatation has been shown to be a strong predictor of intestinal-derived sepsis. Moreover, a significant decrease in the frequency of sepsis has been seen after bowel lengthening and tapering procedures. [16] It has also been reported that half of PN-dependent SBS patients with approximately 20% of the age-adjusted length of the small bowel and SIBO showed a seven times higher incidence of septic infections when compared to SBS patients without SIBO. The main pathogens of the total number of infections were gram negative *Klebsiella pneumonie* (35%), gram positive *Enterococcus faecalis* (25%) and coagulase-negative staphylococci (15%), while 20% were mixed infections and also included *Escherichia coli* [85].

3.4.4 Metabolic complications

PN predisposes to metabolic bone disease where the decreased bone density eventually leads to osteoporosis or osteomalacia [9]. The pathogenesis is poorly understood and may relate secondarily to the IF, to the side effects of the treatment, D-vitamin and calcium deficiency [9,86,87]. In a previous study of PN-dependent IF patients, half of them were short (height SD score <-2) and 42% presented with low metabolic bone density (SDS<-2.0).The risk for fractures seemed to be comparable with the general population and no pathological fractures were reported. [87] In another study, half of the patients showed a mineral bone density Z-score of <-1.0 and almost 20% of the patients suffered from bone pain and 11% of them experienced pathological fracture. In addition, deficiency of the

25-hydroxyvitamin-D occurred in 64%, and hyperparathyroidism in 25%, of the patients. The low bone density and 25-hydroxyvitamin-D levels seemed to persist despite higher oral D-vitamin supplementation [86].

Pediatric IF patients are at risk of developing impaired renal function which grows with prolonged PN-delivery and is associated with a shorter remaining length of the small bowel. Approximately one third (29%) of IF patients showed impaired renal function. [88] Nephrocalcinosis and/or abnormal renal echogenicity might relate to treatment with PN, use of diuretics and vitamin D supplementation, fat malabsorption and episodes of dehydration [89]. In a previous study, of 56 pediatric IF patients, 24 demonstrated nephrocalcinosis/echogenicity and among them, 74% had SBS, 22% dysmotility disorder and 4% enteropathy. The condition was associated with a shorter remaining large bowel, presence of stoma, and long PN-dependency [90].

3.5 Surgical treatment

The main objective of operative interventions is to minimize the extent of bowel resection and to restore bowel continuity as early as possible [7,91]. It enables each remaining intestinal segment to take part in digestion and absorption, improving facilities for the adaptation process [91]. The risks of markable resection of the intestine increase with complex gastroschisis, midgut volvulus (due to ischemic bowel), and jejunoileal atresia [92-94]. NEC, at its early stage, can be treated conservatively in the majority of cases (60-80%), while the most severe form, which affects the entire small bowel with or without other viscera, demands an aggressive surgical approach and extended bowel resection to improve survival rates [49,95].

After primary surgery, patients with SBS require parenteral supplementation to maintain a normal nutritional state while the remaining intestine goes through adaptation. Enteral nutrition should be introduced as soon as possible to facilitate and encourage the adaptation process in the remaining intestine. [96] A significant amount, approximately 18-30%, of children with SBS fail to reach enteral autonomy [3,10,48]. Patients with permanent dependence on PN, without progression in weaning off, accompanied by small bowel dilatation may be considered for undergoing autologous intestinal reconstruction (AIR) procedures to improve bowel function [9,47,91,97]. The most common AIR

procedures are longitudinal intestinal lengthening and tailoring (LILT) and serial transverse enteroplasty (STEP) [91,97].

Bianchi first described the LILT procedure in 1980 [98]. According to his description, the dilated bowel is divided longitudinally into two halves from the avascular space between the anterior and posterior mesenteric vessel layers. Then both separated bowel halves with their own blood supply are anastomosed in isoperistaltic fashion.

Kim *et al.* presented the STEP procedure in 2003 [99] in which the dilated bowel segment is lengthened using a serial transverse gastrointestinal anastomosis stapler. This is adopted in a serial transverse manner from opposite directions, making a zig-zag- shape channel with a diameter of approximately 2-2.5 cm.

Various techniques have been developed to enhance intestinal adaptation by slowing transit time and reducing bowel dilatation. They include artificial intestinal valves, tapering enteroplasty, plication of the antimesenteric bowel, and interposing a part of the colon between two segments of the small bowel. However, data on outcomes is sparse due to limited use. [47,100]

Intestinal transplantation is considered to be important for patients with irreversible intestinal failure and who suffer from life-threatening complications due to total PN. Such complications include recurrent sepsis, frequent central line catheter infections, absence of intravenous access, and parenteral nutrition-associated liver disease. Intestinal allograft is a small intestine graft with or without the colon. Other multivisceral allografts are small intestine with a liver graft, small intestine with a stomach graft, or small intestine with a stomach and liver graft. All grafts may include a segment of the colon regarding the underlaying disease indication. [101] One-year and five- year survival after intestinal transplantation in children is 73 and 57%, respectively. Graft loss occurs mostly due to rejection and death caused by sepsis. [102] Patients need high-immunosuppression requirements and accurate surveillance as heavy medication is likely to predispose to severe metabolic complications [102,103].

3.6 Bowel adaptation

A successful bowel adaptation enables patients with SBS to wean off PN [3,96]. During adaptation, the intestine modifies to new conditions by altering its structure and hormonal responses [14,17,47,104]. Both the small and large bowel take part in this delicate process [14,17,105,106]. A large number of studies with animal SBS models show a prompt response to notable small bowel resection [12,14,15,18-20,107]. The intestinal adaptation includes the lengthening of villi and deepening of the crypts accompanied with lengthening, thickening and dilatation of the remaining bowel, which increases the absorptive capacity of the intestine [14,17,47].

3.6.1 Crypts and villi

Epithelial cell population, which consists of crypt-villus units, is thought to be regulated in the intestine by apoptosis under normal physiologic circumstances [108]. Crypts harbor transit-amplifying cells, which divide four to five times and then differentiate into intestinal epithelial cells (the absorptive enterocytes, mucous-secreting goblet cells, or hormone-secreting enteroendocrine cells) within three days and migrate from the base of the villi to the top [109]. When these cells fulfil their different functions (brush border enzyme secretion, mucus secretion, nutrient and water absorption), they undergo apoptosis and are dispersed into the intestinal lumen [108-110].

3.6.2 Muscular layer

Longitudinal and circular layers of the muscles in the remaining intestine of rats seem to thicken after the massive resection which is associated with the disturbance of tight junctions and altered membrane permeability. The smooth muscles, which are responsible for large and regular movements in the intestine, showed an irregular motion with an abnormally low amplitude seven days after markable small bowel resection in rats. The animals also showed a significantly higher rate of bacterial translocation when compared with the transection procedure group. [14]

A study of muscular contractions in dogs two weeks after 80% of small bowel resection and laparotomy (unresected controls) revealed that resected dogs showed a significantly longer interval between migrating myoelectric complexes (MMCs) in the duodenum

during a fasting state (mean 153 vs 126 min, P<0.01), as well as the postprandial period (mean 23 h vs 8, P<0.01). At the same time, the propagation velocity of MMCs was significantly slower in resected dogs (mean 1.4 vs 2.3 cm min^{-1}, P<0.01). [111] Prolonged transit time may compensate for the loss of a functioning intestine and improve the absorptive capacity of the remaining small bowel.

3.6.3 Adaptation in the jejunum and ileum

Previous literature demonstrates a greater adaptation ability in the ileum, when compared to the jejunum. This adaptation reflects more extensive villous hypertrophy, crypt elongation, higher epithelial cell production and apoptosis, as well as changes in mRNA expression of genes related to epithelial cell proliferation and apoptosis. [18,19] However, mucosal adaptation occurs also in the duodenum [12,15,20]. It is notable that increases in the length and diameter of the remaining post-resectional small bowel does not always lead to a total increase in the mucosal absorptive surface area. Previously reported pig models with a 75% midgut resection demonstrated a major increase in the bowel length, diameter, and size of the ileal villi. However, the changes were accompanied with a decrease in villous density. Thus, the mucosal surface area per unit of the serosal area remained at the same level as transected animals [112].

Disturbed bile acid homeostasis has been reported in SBS animal models and children [112-114]. SBS children showed extremely high secretion of bile acids into feces and children with severe diarrhea showed incomplete metabolizing of the bile acids [113]. Disturbed glucose homeostasis and elevated serum TNFα were reported previously in mice SBS models ten weeks after major small bowel resection [114]. While, pig SBS models demonstrated a decrease in the ability to absorb fat, protein and carbohydrates despite the increase in remaining intestinal length and enteral nutrition after bowel resection [112].

3.6.4 Adaptation in the duodenum

Data about adaptation mechanisms of the duodenum after major small bowel resection is sparse [12,15,115,116]. Rats studied 14 days after an 80% small bowel resection showed a significant increase in duodenal mucosal mass, mucosal DNA, protein, and sucrase

activity compared to rats with transection procedure (P<0.001 for all) [12]. Similar results occurred in four months old rats after 70% small bowel resections, when duodenal weight and mucosal DNA, RNA and protein content were significantly higher than in transected control animals after 10 and 20 post-operative days (P<0.05 for all) [115]. B-Cell lymphoma (BCL2) Associated X (*BAX)* is the gene encoding protein which belongs to BCL-2 family, the anti- and pro-apoptotic regulators of apoptosis [116]. *BAX* +/+ and -/- mice were studied seven days after 50% small bowel resections, showing a significant increase in duodenal villus height and crypt depth when compared to transected control animals in both genetic models [15].

3.6.5 Adaptation in the colon

The preserved ileum facilitates the adaptation to the enteral autonomy as mentioned before [15,18,19]. A preserved colon also plays an important role in bowel adaptation – it serves as an absorptive field for lipids and carbohydrates, operates re-absorption of water and electrolytes, and minimizes the loss of energy [105,106,117]. Rats with massive small bowel resection, up to 95%, showed major adaptation in the remaining gut within three weeks. It resulted in a significant increase in the thickness of colonic mucosa, the height of colon plica and altered protein expression. [118] Moreover, the bacterial fragmentation of indigestible foods led the colon to produce short chain fatty acids (SCFAs), mainly acetate, propionate, and butyrate. These serve as an energy supply for colonocytes (butyrate), participate in the maintenance of epithelial integrity and cell cycles (proliferation, differentiation, apoptosis), fluid absorption, and play an important role in hormonal secretion and anti-inflammatory responses. [119] A major small bowel resection decreases the colonic SCFAs content which may lead to altered mucosal homeostasis through the actual loss of SCFA receptors. For instance, activation of free fatty acid receptors two and three has been linked to reduced expression of proinflammatory cytokines *in vitro* studies and has shown a protective effect on the intestinal mucosa. Loss of these receptors may eventually lead to an increase in inflammation and disturbed permeability. [119,120] Small intestinal resection was reported to be associated with higher absorption of the long chain fatty acids in the remaining bowel, which in turn, may facilitate intestinal adaptation [121].

3.6.6 Hormonal factors

In addition to the distal intestine as a part of bowel adaptation, the secretion of glucagon-like peptide-2 (GLP-2) is another important factor [122]. A distal ileum and colon contain most of the intestinal GLP-2 producing enteroendocrine L cells, which are activated by nutrient exposure [123,124]. The GLP-2 acts in several ways in the intestine: it enhances crypt cell proliferation, reduces epithelium apoptosis, increases blood flow and nutrient absorption, and also reduces intestinal motility, permeability, inflammation and gastric acid secretion [123]. To assess the impact of luminal nutrients and the GLP-2 system on adaptation, some of the ileum and intact colon was left in continuity after major small bowel resection in rats. Dahly *et al*. thus reported that an intestinal adaptation occurs due to resection itself, even under total parenteral nutrition (TPN) conditions in rats. The animals showed significant mucosal hyperplasia in the duodenum, jejunum and ileum, while transected animals on TPN showed significant jejunal hypoplasia. As expected, the resected rats on enteral nutrition also showed mucosal hyperplasia in all remaining small bowel segments with a significant difference when compared to the transected group. As a result, the exposure to enteral feeds was associated with elevated levels of ileal (but not colonic) proglucagon mRNA and plasma bioactive GLP-2 in the resected animals. [107] Mutanen and Pakarinen reported higher serum GLP-2 levels in pediatric IF patients, regardless of the nutritional status, in comparison to healthy individuals. The GLP-2 levels were higher in patients with the colon in continuity. [125] These findings support the theory of a positive effect of a remaining colon on intestinal adaptation [107,125].

Exogenous GLP-2 can be successfully used to enhance adaptation after bowel resection [126-132]. Treatment with teduglutide (GLP-2 analogue) after 80% jejunoileal resections in piglets on TPN and partial PN showed significant increases in villous height of all the remaining small bowel segments and crypt elongation of the ileum and colon when compared with the vehicle group. The study reported a significant increase in epithelial cell proliferation and decrease in apoptosis in both the small and large bowel, with changes in groups on partial enteral nutrition being more significant. Glucose and glutamine transport were enhanced four hours post-resectionally. [126] In line with these findings, enterally fed SBS rat models treated with teduglutide after 70% jejunoileal resections showed a significant increase in villous height, ileal crypt cell proliferation,

increased sucrase activity and GLP-2 receptor expression in the remaining small bowel [127]. In contrast to these studies, enterally fed piglets with jejunostomy showed no morphological changes in the remining intestine, no enhances in digestive enzyme activity or absorption of enteral nutrients after 50% small bowel resections in comparison to a placebo group [128]. Two studies on adult SBS/ IF PN dependent patients treated with teduglutide for three and 24 weeks showed significant increases in small bowel villous height and crypt depth [129,130]. A twelve-week clinical trial on pediatric SBS showed that treatment of PN dependent patients with teduglutide doses 0.025 or 0.05 mg/kg/day tended to allow reductions in PN support and improved enteral nutrition feeding [132]. Teduglutide is now approved by the European Union for the treatment of PN dependent adults and children from the age of ≥1 years with SBS [131].

3.6.7 Enteral nutrition and disaccharidase activity

During PN or fasting, the intestinal mucosa demonstrates atrophy and a decreased level of disaccharidase activities in both resected and intact small bowels in humans [133-135]. Enteral nutrition is well-known to promote intestinal adaptation in SBS children and should be started as soon as possible after bowel resection [104,136,137]. It was reported that early enteral feeding in small volumes accelerates the time to a first stool, the adaptation to a full oral intake and being discharged from hospital in neonates after abdominal surgery [137]. The question then becomes how oral food enhances intestinal adaptation. Enteral nutrition serves as a source of glutamine, carbohydrates and lipids among other nutrients [136]. Glutamine is an important amino acid which works as a major fuel for the mitochondrial respiration of small bowel mucosal enterocytes [138]. The levels of plasma glutamine are thought to reflect the enterocyte mass in the intestine [139]. Glutamine effectively activates L-cells in order to produce another hormone, GLP-1, which is partly mediated by a solute carrier protein (SLC38A2) family [140]. The GLP-1 hormone enhances insulin secretion via glucose-dependent stimulation [141]. In addition, the growth hormone (rhGH) was shown to improve glutamine synthesis and treatment with rhGH was reported to increase the glutamine availability, the body weight, the lean body mass and the absorptive capacity of SBS patients [139,142]. A precise mechanism of glutamine on the adaptation of the remaining bowel is still to be clarified [143]. Dietary supplementation of glutamine failed to show any beneficial effects on post-

resectional bowel adaptation [139]. However, rat SBS models with 80% small bowel resections were able to reach the same level of glutamine intake by the remaining small bowel as a sham-operated control group within 24 hours post-resectionally. This highlights the importance of glutamine during the adaptation process [143].

Carbohydrates are macronutrients with water-soluble components which require specific channels in the luminal side of the villi for absorption. Small intestinal mucosal levels of brash border enzymes such as maltase, sucrase and lactase change depending on the dietary supply of their substrates. Moreover, oral diet modifies the transcriptional control of transporters such as Sodium/Glucose Cotransporter 1 (SGLT1) and Fructose Transporter (GLUT5). Both are required for the utilization of monosaccharides. [144] The marked small bowel resections in the experimental animals receiving enteral nutrition have been shown to be associated with various activities of brush border enzymes in the remaining intestine (increased, unchanged and decreased activities) [145-147]. Laffolie *et al*. researched disaccharidase activities in the duodenum and colon in pediatric SBS patients, who mostly demonstrate a medium or low activity, compared to controls and normal values. The patients with higher levels of disaccharidase activities either in the duodenum or colon seemed to demonstrate higher proliferation in crypts in the corresponding part of the bowel. [20] The impact of small bowel resection on the amount of digestive enzymes may also be mediated by other factors, such as a faster migration of immature enterocytes to the top of the already elongated villi, which increases the distance during the migration. On the other hand, a significant loss of intestinal mucosa may influence the maturation process of enzymes on the villus [145,147].

To conclude, intestinal adaptation is a multifactorial process which is regulated by humoral factors and results in significant changes within the remaining intestine, allowing patients to wean off PN [7].

AIMS OF THE STUDY

The goal of this work was to investigate the adaptation of the duodenum after significant small bowel resection during current PN-dependence and after achieving full enteral autonomy in pediatric SBS.

The specific aims were:

1) To evaluate disaccharidase activities for maltase, sucrase and lactase, and assess the level of histological inflammation in the duodenum during and after weaning off PN in patients with pediatric intestinal failure. (Study I)
2) To study duodenal biopsies to evaluate mucosal microarchitecture, proliferation, apoptosis, inflammation, and epithelial-barrier function using histology, immunohistochemistry, and qPCR during and after weaning off PN children with SBS. (Studies II-III)
3) To assess the effects of pathological dilatation of the remaining small bowel and removal of ileocecal valve on structural hyperplasia, proliferation, apoptosis, inflammation and gene expression of duodenal mucosa in patients with SBS after adaptation to enteral autonomy. (Study III)

METHODS

1 Ethics (I-III)

This work was approved by the Helsinki University Hospital (Helsinki, Finland) Ethics Committee (no. 2/13/03/03/2010 for I-III) and the Institutional Review Board (no. 67/2017, no. 57/2010, no. 12/2013 for I-III, respectively). Informed written consent was received from all patients and controls participating in these studies and/or caregivers before any procedures (I-III).

2 Patients

All included patients (I-III) were treated in the IF rehabilitation program of the Helsinki University Children's Hospital [3]. Duodenal biopsies obtained during gastroscopies performed as part of clinical patient follow-up were collected. Patients without available duodenal biopsies or with poor-quality biopsy specimens were excluded. We collected clinical patient data from patient records, including demographics, intestinal resections, other surgical procedures, anatomy of remaining intestine, and the duration of PN. The percentage of predicted age-adjusted length of the small bowel was calculated according to published age-specific normal values [23]. The percentage of the remaining large bowel was measured according to Mitchell and his colleagues, where it is divided in arbitrary manner into 14 equal sections, each one representing seven percent of the large bowel [106]. Hirschsprung disease patients with a remaining small bowel length < 50% of expected were considered SBS patients. In order to assess the degree of dilation in the remaining small bowel, the maximum small bowel width perpendicular to the longitudinal axis of the remaining small bowel and the height of the fifth lumbar vertebra were measured in the same contrast small bowel series. Dilatation is reported in millimeters and in relation to the height of the fifth lumbar vertebra (small bowel diameter ratio; SBDR) to take in account variable age and physical size of the patients. SBDR >2 was considered pathological [148].

Study I

Measurements of disaccharidase activities and assessments of the original pathology reports were carried out for duodenal samples obtained between 1999 and 2015. We included 58 patients with SBS (n=49) and dysmotility disorders (n=9), of whom five underwent gastroscopy both during PN and after weaning off PN.

Studies II and III

We included 14 children with duodenal biopsies obtained while receiving long-term PN (II), and 33 children who had weaned off PN and achieved enteral autonomy (III), to study duodenal mucosal microarchitecture, proliferation, apoptosis, inflammation and epithelial barrier function. Individuals with an underlying primary motility disorder or mucosal enteropathy were excluded [3].

3 Controls (I-III)

Duodenal biopsies of generally healthy age- and sex-matched children without gastrointestinal pathology served as normal controls. Control individuals underwent gastroscopy for different symptoms, such as respiratory symptoms (uncontrolled or partially controlled asthma despite treatment, mostly with a suspicion of gastroesophageal reflux and/ or abdominal symptoms), gastroesophageal reflux symptoms, chronic abdominal pain, or dysphagia. There were no diagnostic macroscopic findings in the gastroscopy or in the pathologic analysis and thus, biopsies were considered normal samples according to previous literature [149].

4 Duodenal biopsies (I-III)

All patients and controls underwent gastroscopies and biopsies after an overnight fast under general anesthesia by an experienced pediatric surgeon or gastroenterologist. The biopsy sections were fixed in formalin, embedded in paraffin, sliced and stained with hematoxylin and eosin (H&E) for further analysis. The biopsies for disaccharidase activity measurements were immediately embedded in ice and frozen until analyzed (I).

For studies II and III, duodenal biopsy specimens were embedded in RNAlater (Ambion, Life technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA).

5 Disaccharidase analysis (I)

Duodenal biopsies were first homogenized and incubated with different substrates for maltase, sucrase and lactase. The enzyme activities were subsequently determined by measuring the amount of released glucose using the glucose oxidase method as described in previous studies [150,151]. Enzyme activities are reported as units of substrate hydrolyzed per minute at 37°C per gram of protein (U g^{-1} of protein) and are not age adjusted. The reference values used in our hospital for maltase were 150-700 U g^{-1} of protein, for sucrase 40-250 U g^{-1} of protein, and for lactase 20-140 U g^{-1} of protein [152].

6 Histological analysis (I-III)

Study I

H&E stained slices were analyzed by an experienced pediatric pathologist. The presence of an abnormal inflammatory infiltrate was considered mucosal inflammation. Mucosal inflammation or its absence, or any other abnormalities, in mucosal architecture and epithelial lining were recorded from the original pathology reports.

Studies II and III.

H&E slices (III, Fig1 A, B) were first reviewed manually using a light microscope and then photographed. More detailed descriptions are reported in the Methods section of articles II and III. Samples with well-oriented villi and crypts were included and evaluated for villus height and width, crypt depth (Fig 4), apoptosis index (Fig 2B), and inflammation.

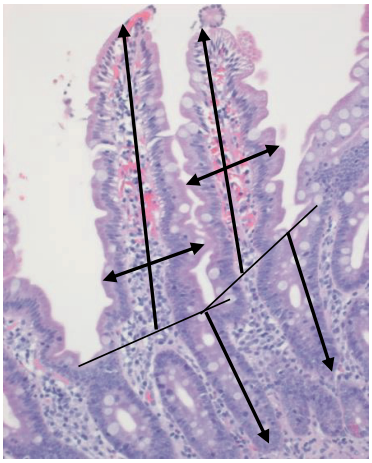


Figure 4. Measurements of villus height and width, and crypt depth.

A median of 9 (6-11) (study II) and 11 (8-14) (study III) representative villi, and 8 (4-12) (study II) and 9 (6-17) (study III) representative crypts were assessed for each patient. To assess apoptosis, the crypts were first manually analyzed for apoptotic bodies (Fig 2 B) and the ten crypts with the highest apoptotic activity were chosen and photographed. The apoptotic index includes the amount of apoptotic bodies per ten well-oriented crypts [153]. Inflammation in the lamina propria (III, Fig 1 C,D) was evaluated semi-quantitatively (grade 1 = few loosely spaced and scattered inflammatory cells between crypts; 2 = moderate number of inflammatory cells spaced closely to each other and distributed diffusely throughout the lamina propria, 3 = large number of inflammatory cells close to each other, distributed diffusely through the lamina propria, often in combination with short or broad villi; 4 = intense/very heavy infiltrate of tightly spaced inflammatory cells across the lamina propria with short, broad or absent villi) as previously described [154,155]. The number of intraepithelial leukocytes such as lymphocytes, eosinophils and neutrophils were counted in three representative villi per 100 enterocytes [156].

7 Immunohistochemistry (II-III)

A detailed description of immunohistochemistry methods is shown in the Methods-section of articles II and III. The antibodies are summarized in Table 3. Mucosal

proliferation was measured using MIB-1 monoclonal antibody (Immunotech, Marseilles, France) against nuclear cell proliferation-associated Ki-67 antigen from microwaved formalin fixed, paraffin-embedded sections [157]. All sections were first evaluated manually, then the three most representative and well-oriented villi and crypts were chosen for analysis. To calculate the MIB-1 proliferation index, the number of positively stained enterocytes was counted and expressed as a percentage of the total number of enterocytes in each villus and crypt (III, Fig 1 E,F) [158]. Overall MIB-1 proliferation grade was assessed semi-quantitatively from 1 to 3 based on the location of positively stained enterocytes along crypt-villus-axis (grade 1 = positive enterocytes in crypts, 2 = positive enterocytes in crypts and the lower half of the villi, 3 = positive enterocytes in crypts and above the lower half of the villi). For the mucin-2 ratio, the number of mucin-2 stained villus goblet cells in relation to the villus length was calculated (III, Fig 1 G, H).

Table 3. List of antibodies used in studies II and III.

Antibody	Manufacturer	City, country	Dilution	Classification	Host species
Mucin-2 (MUC2)	Abcam	Cambridge, UK	1:15 000	Monoclonal	Rabbit
Caveoli-1 (CAV1)	Abcam	Cambridge, UK	1:25 000	Polyclonal	Rabbit
MIB-1	Immunotech	Marseilles, France	1: 100	Monoclonal	Mouse

8 Messenger RNA expression analysis (II-III)

Mucosal messenger RNA (mRNA) expression was analyzed in 11 patients on current PN, 25 weaned-off patients and in all controls (n=6 in study II and n= 12 in study III). Duodenal biopsy specimens were embedded in RNAlater (Ambion, Life technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA) and frozen until analyzed. RNA was then extracted using the RNeasy Mini Kit (QIAGEN, Frederick, MD, USA). The assessment of RNA concentration was done spectrophotometrically. mRNA expression of various genes regulating cell cycle, inflammation, epithelial permeability, and nutrient transport (Table 4) were analyzed in triplicate by quantitative real-time polymerase chain reaction using a Custom RT Profiler PCR Array (CAPH12366A) (QIAGEN SABiosciences, Frederick, MD, USA) on an ABI 7700 Sequence Detection System

(Perkin-Elmer Life Sciences, Boston, MA, USA) according to the manufacturer's instructions. The genes used in these studies were chosen based on previous literature (Table 4) and HPRT1, RPLP0, ACTB, B2M, and HSP90AB1 were used as housekeeping genes. Quantification of target gene mRNA expression was performed using the ΔΔCt method and expressed after normalization to housekeeping genes and relative to control individuals after normalization.

9 Statistical analysis (I-III)

Statistical analysis was performed with Statistical Package for the Social Sciences Software (SPSS version 23.0Inc./IBM, Finland for study I and SPSS version 25.0Inc./IBM, Finland for studies II-III). Descriptive statistics are reported as medians with interquartile range (IQR) or frequencies, except for the mRNA expression (mean and IQR). Due to small number of patients and controls we chose non parametrical tests to assess the data. For comparisons between groups we used Fisher`s exact and Mann-Whitney U tests, for correlations Spearman rank correlation test and for identifying predictors for disaccharidase activities single and multiple linear and logistic regression models were performed. Statistical significance in this work was considered at $P< 0.05$.

Table 4. Table represents in studies II and II analyzed genes, their products and main functions.

Group/ Gene	Product	Function	Reference
Inflammation/cytokines			
TNF	Tumor necrosis factor	Inducing occludin removal from caveolar endocytosis, which leads to a loss of tight junction function. Activating myosin light chain mediated pathway, thus increasing tight junction permeability.	[40,159]
IFNG	Interferon-γ	T-helper type 1 cytokine, acting against attaching-and-effacing pathogen. Might participate in pathogenesis of inflammatory bowel diseases. Increasing membrane permeability.	[159]
IL1A	Interleukin 1α	Cytokine. Participating the influx of immune cells into the mucosa, epithelial cell growth and intestinal homeostasis.	[160]
IL1B	Interleukin 1β	Cytokine, which is upregulated during microbial infection. Increasing in membrane permeability.	[159,161]
IL6	Interleukin 6	Cytokine. Participating the influx of immune cells into the mucosa, epithelial cell growth and intestinal homeostasis. Increasing membrane permeability.	[159,160]

IL8	Interleukin 8	Cytokine, which is upregulated during microbial infection, involved in the development of inflammatory T-helper 1 lymphocyte responses, increasing mitotic activity.	[160,162]
IL10	Interleukin 10	Anti-inflammatory cytokine, mediating of downregulation of monocyte/macrophage activation and inducing lymphocyte differentiation. Can suppress the secretion of IL-1β and TNF-α, can decrease membrane permeability.	[159-161]
IL17A	Interleukin 17α	T-helper 17 lymphocytes acts via secretion of cytokines such as proinflammatory cytokine IL-17A. Functioning against bacterial and fungal infections and contributing to inflammatory diseases, decreasing membrane permeability and inducing inflammation.	[159,163]
IL18	Interleukin 18	Proinflammatory cytokine, produced by monocytes/macrophages and keratinocytes. Participating in T cell development, increasing IFN-gamma expression.	[164]
TLR2	Toll-like receptor 2	Transmembrane receptor on intestinal epithelium cells, responding to bacterial products (lipoproteins, lipopolysaccharide, flagellin). Inducing expression of inflammatory cytokines (such as IL-6, TNF-α) or anti-inflammatory responses (via IL-10).	[165,166]
TLR3	Toll-like receptor 3	Intracellular receptor, localized in endosome, responding to nucleic acid. Inducing inflammatory cytokines such as IL-6, TNF-α.	
TLR4	Toll-like receptor 4	Cellular membrane receptor inducing pro-inflammatory signaling (cytokines like IL-6, TNF-α), intracellular receptor anti-inflammatory signaling.	
TLR5	Toll-like receptor 5	Transmembrane receptor on intestinal epithelium cells, responding to bacterial products (lipoproteins, lipopolysaccharide, flagellin). Inducing inflammatory cytokines such as IL-6, TNF-α.	
TLR8	Toll-like receptor 8	Intracellular receptor, localized in endosome, responding to nucleic acid. Inducing inflammatory cytokines such as IL-6, TNF-α.	
TLR9	Toll-like receptor 9	Intracellular receptor, localized in endosome, responding to nucleic acid. Inducing inflammatory cytokines such as IL-6, TNF-α.	
CASP1	Caspase 1	Inducing activation and secretion of proinflammatory cytokines IL1β and IL-18, activation of pyroptosis.	[167]
CASP4	Caspase 4	Inducing pyroptosis by detecting lipopolysaccharide in monocytes.	
TGFB1	Transforming growth factor β1	Cytokine. Prolonging or arresting the epithelial cell cycle in the G1-phase (reduction of proliferation and early apoptosis). Interaction with CAV1-signaling. Regulating immune function, epithelial cell growth, differentiation and intestinal homeostasis. Decreasing membrane permeability.	[168,169]
TGFB2	Transforming growth factor β2	Cytokine. Interaction with CAV1-signaling. Regulating immune function, epithelial cell growth, differentiation and intestinal homeostasis Decreasing membrane permeability.	

Epithelial barrier function			
HP	Haptoglobin, Zonulin	Activation of EGFR. Secretion from intestinal mucosa after exposure to gluten or microorganisms.	[170]
HPR	Haptoglobin-related protein	*HPR* is a part of HP-gene in chromosome 16.	
OCLN	Occludin	Forming tight junction protein complexes with Claudin, able to bind to junctional adhesion molecule 1.	[36,40,159,171]
CLDN1	Claudin 1	Forming tight junction protein complexes with occludin. Claudins interact with adjacent cells formatting barriers or pores in the membrane for the passage of selective molecules.	
CLDN2	Claudin 2		
CLDN3	Claudin 3		
CDH1	Cadherin 1	A component of tight junctions, promoting intestinal homeostasis and barrier function.	[38,159]
MUC2	Mucin 2	Main gel-forming mucin of small and large intestines.	[37]
F11R	Junctional adhesion molecule 1	Member of immunoglobulin superfamily proteins of tight junctions of epithelial and endothelial cells, able to bind to other proteins, for example occludin.	[159,172]
FGF7	Fibroblast growth factor 7	Keratinocyte Growth Factor, KGF or FGF7, expressed in mucosal layer by intraepithelial lymphocytes. Improving barrier function.	[171]
CAV1	Caveolin 1	Caveolin 1 localized in plasma membrane invaginations. Interaction with TGFB-signaling.	[168,169,173]
NLRC4	NLR family CARD containing 4	Inflammasome, activating of Caspase-1, responding to bacterial proteins.	[167]
Apoptosis/proliferation			
BAX	BCL2 associated X	Member of the bcl-2 family, able to prevent cell death.	[15,174]
BCL2	B-Cell lymphoma 2	External mitochondrial membrane protein, direct regulation of permeability of the membrane, is able to block the apoptotic death (for example in lymphocytes).	
NAIP	NLR family apoptosis inhibitory protein	Responding to type III secretion system needle protein (mediating of bacterial effectors to the host cytosol for detecting by NLRC4).	[167]
NLRP1	NLR family pyrin domain containing 1	Inflammasome, responding to bacterial infections and inducing pyroptosis. Partly dependent on IL-18.	
NLRP3	NLR family pyrin domain containing 3	Sensation of fungal, bacterial, viral pathogens, pore-forming toxins and crystals.	
NLRP6	NLR family pyrin domain containing 6	Controlling mucus release from Goblet cells.	
PCNA	Proliferating cell nuclear antigen	Encodes cofactor of DNA polymerase delta protein, participating in cell proliferation.	[171]
MKI67	Marker of proliferation Ki-67	Ki-67 protein is involved in structural and functional rearrangements during mitosis.	[175]
GCG	Glucagon		[176]
ZGLP1	Glucagon like peptide 1	Enhancing insulin secretion via glucose-dependent stimulation.	

GLP2R	Glucagon like peptide 2 receptor	GLP-2 functions are mediated by GLP2R. Stimulation of nutrient absorption, crypt cell proliferation and decrease of apoptosis.	
EGF	Epidermal growth factor	Transmembrane protein. Regulation of cellular proliferation, differentiation and survival, decreasing membrane permeability through EGFR. Protection of barrier function against oxidative stress, ethanol and acetaldehyde.	
EGFR	Epidermal growth factor receptor		
Nutrient transport			
ABCG5	ABCG5, Sterol transporter	ABCG5/G8 mRNA localized in hepatocytes and enterocytes, cholesterol homeostasis.	[177,178]
ABCG8	ABCG8, Sterol transporter		
NPC1L1	Niemann-Pick C1-Like 1, Sterol transporter	Transmembrane transporter, absorption of cholesterol from the intestine, mediating absorption of biliary cholesterol.	[178,179]
FABP2	Fatty acid binding protein	Participating in fatty acid entry into mitochondria.	[178,180]
SLC27A4	FATP4, Fatty acid transporter	Integral membrane protein, might be able to drive fatty acid uptake or activate fatty acids by trapping them into the cell.	[178]
SLC5A1	SLGT1, Glucose transporter	Participating in transepithelial transport of glucose together with GLUT2 and SLC5A2.	[40,181]
SLC2A1	GLUT1, Glucose transporter 1	Membrane transporter, transporting glucose, galactose, mannose and glucosamine into the cell.	[181]
SLC15A1	PEPT1, Peptide transporter 1	Enterocyte plasma membrane transporter for di- and tripeptides.	[182]

ABCG, ATP binding cassette subfamily G; ATP, adenosine triphosphate; CARD, caspase recruitment domain; FATP4, long-chain fatty acid transport protein 4; NLR, Nod- like receptor; SLGT1, Sodium/Glucose Cotransporter 1

RESULTS

1 Patient Characteristics (I-III)

Baseline patient characteristics are summarized in Table 5. In studies I-III, the median age at the time of gastroscopy varied between 1.5 and 5.5 years and the median duration of PN from 0.9 to 2.2 years. In SBS patients, the median length of the remining small bowel was 33-40 cm or 20-29% of expected, 43-48% of patients had a preserved ileocecal valve, and 0-14% had enterostomy. The underlying causes for SBS included NEC, volvulus, SBA and gastroschisis with or without SBA. None of the participants had received any intestinotrophic therapy (GLP-2 analogues or growth hormone) prior to the study. For studies II and III, we identified patients who received antimicrobial therapy for any reason (six months in study II and two months in study III) prior to the gastroscopy (Table 5).

Table 5. Baseline characteristics and remaining bowel anatomy of patients (studies I-III).

	Study I		Study II	Study III
Variable	SBS patients	Patients with dysmotility disorder	SBS patients on PN	SBS patients weaned off PN
	n=49	n=9	n=14	n=33
Baseline characteristics				
Age (years)	3.0 (1.1-7.0)	5.5 (3.4-15.5)	1.5 (1.0-6.5)	4.7 (2.3-13)
Gender (male n/%)	27/55	6/68	9/64	20/59
Duration of PN (years)	1.8 (0.8-4.2)	2.2 (0.9-6.8)	1.4 (0.7-6.5)	0.9 (0.4-2.0)
Time after weaning off PN (years)	2.6 (0.8-6.9)	3.3 (2.6-)	N/A	3.5 (0.8-8.5)
Time after last bowel resection (years)	2.8 (1.0-5.0)	3.0 (0.8-15.3)	1.4 (0.4-6.9)	4.1 (1.9-10.9)
Antimicrobial therapy n/%	NR	NR	12/86	11/32
Remained bowel anatomy, small bowel diameter and ratio				
SB (cm)	40 (25-60)	225 (179-305)	33 (12-60)	47 (30-60)
SB (% of expected)	25 (16-35)	88 (84-100)	20 (9-22)	29 (19-43)
Ileum (cm)	5 (3-10)*	66 (48-80)**	0 (0-5)	0 (0-5)
ICV preserved (n/%)	23/47	4/44	6/43	16/48
Large bowel (% of expected)	86 (57-100)	0 (0-100)	67 (27-100)	80 (60-100)
Enterostomy (n/%)	7/14	5/56	5/36	0/0
SBD (mm)	NR	NR	NR	27 (20-37)[1]
SBDR	NR	NR	NR	1.7 (1.4-2.2)[2]
Diagnosis (n/%)				
NEC	21/43	0	4/29	16/48
Volvulus	8/16	0	5/36	7/21
SBA	4/8	0	0	4/12
Gastroschisis w/wo SBA	5/10	0	2/14	2/6
Dysmotility disorders	8/16	8/89	3/21	4/12
PIPO with SBA	0	1/11	0	0

Data expressed as median and IQR or frequencies. [1]n=28 ; [2]n=27 ; *=in patients with preserved ileum (n=24); **= in patients with preserved ileum (n=4); PIPO, pediatric intestinal pseudo obstruction ; EN, enteral nutrition ; NR, not reported ; PN, parenteral nutrition ; SBA, small bowel atresia ; SBD, small bowel diameter ; SBDR, small bowel diameter ratio. Dysmotility disorder with remained small bowel length of <50% of expected considered as SBS.

2 Disaccharidase activities and mucosal inflammation (I)

We evaluated biopsies obtained at two time points: closest to and furthest from the latest intestinal resection (early and late, respectively). Patients on PN were significantly younger than patients on full enteral nutrition (median 3.5 years vs 5.2, $P<0.005$) at the early time point but not at the late time point (4.9 vs 6.8, $P>0.05$). The activities of maltase and sucrase were significantly lower in patients on PN when compare to the matched control group or weaned-off patients at both timey points. Patients on PN also showed

abnormal histology and presence of inflammation more often than matched controls at the late study point. Lactase activity was comparable between all groups at both study points. All disaccharidase activities were similar between patients on full enteral nutrition and matched controls (Table 6).

Among all patients on current PN studied at the late study point [duration of PN median (IQR) 50 (12-110) months and time after last intestinal resection median (IQR) 3.4 (0.8-9.1) years], 22% (n=5) showed some inflammation and 30% showed abnormalities in histology (Table 6).

Table 6. Disaccharidase activities and histology in patients on PN, weaned-off patients and matched controls (I).

Variable	Study point	Patients on PN	Weaned-off patients	Controls (for patients on PN)	Controls (for weaned-off patients)
Number of patients	E	23	40	25	34
	L	23	40	34	39
Maltase (U/of protein)	E	213 (136-277)*	300 (195-390)†	302 (210-238)	307 (212-402)
	L	187 (94-302)*	285 (195-367)†	307 (212-402)	302 (213-390)
Sucrase (U/g of protein)	E	55 (29-70)*	72 (55-99)†	72 (48-101)	74 (48-116)
	L	47 (26-78)*	69 (54-99)†	74 (48-116)	74 (51-116)
Normal histology n (%)	E	21 (91)	35 (88)	23 (92)	32 (94)
	L	16 (70)*	35(88)	32 (94)	36 (92)
Duodenal inflammation n (%)	E	1 (4)	4 (10)	1 (4)	1 (3)
	L	5 (22)*	2 (5)	1 (3)	1 (3)

Data expressed as are median and IQR, comparisons made using Mann Whitney U and Fisher Exact tests. †P<0.05 vs patients on PN, *P<0.05 vs respective control group. Fifty-eight patients underwent gastroscopy, of them five were studied separately before and after achieving total enteral autonomy. Time points: E (early), closest to and L (late), furthest from the last intestinal resection; PN, parenteral nutrition.

When all 63 biopsies from 58 patients were included, all disaccharidase activities correlated positively with the percentage of the remaining colon (I; Table 3). The correlations with statistical significance between bowel anatomy and disaccharidase activities during PN and after weaning off PN at different time points are summarized in Table 7. In addition, correlations between the anatomy of each part of the remaining bowel and disaccharidase activities at both study points are shown in study I, Table 3. The remaining percentage length of the small bowel was inversely related to duodenal maltase and sucrase activity after weaning off PN. The remaining absolute length of ileum had a positive correlation with lactase activity, both during and after weaning off PN.

Table 7. Correlations with significant findings between remained bowel anatomy and disaccharidase activities (study I).

	Maltase	Sucrase	Lactase
Parenteral nutrition, late time point			
Ileum (cm)	r=0.347 P=0.104	r=0.307 P=0.154	**r=0.488** **P=0.018**
Colon (% of expected)	**r=0.424** **P=0.044**	r=0.348 P=0.104	**r= 0.544** **P=0.007**
Enteral nutrition, early time point			
Small bowel length (% of expected)	**r= -0.337** **P=0.034**	**r= -0.407** **P=0.009**	r=-0.201 P=0.214
Enteral nutrition, late time point			
Ileum (cm)	r=-0.015 P=0.929	r=0.023 P=0.888	**r=0.368** **P=0.021**

3 Structural mucosal morphology, proliferation, apoptosis and inflammation (II, III)

Patients currently dependent on PN and weaned-off patients showed similar results (P-value >0.05 for all) in the evaluation of duodenal morphology, proliferation and apoptosis in relation to matched controls (Table 8). Patients who had weaned off PN with a small bowel length <20% of expected (n=8) showed significantly longer villi than those (n=25) with a longer remaining small bowel [median (IQR) 871 (663-981) vs 681 (544-792) µm, P=0.048]. Villus height, however, remained comparable to controls [median (IQR) 871 (663-981) vs (810 (665-946) µm, P=0.616)]. While percentage of the remaining small bowel length inversely correlated with villus height (r=-0.397, P=0.022), patient age was not related to villus height (r=0.143, P=0.746) like it was in controls (r=0.734, P=0.007).

Inflammation of lamina propria was increased in patients who had weaned off PN, while intraepithelial leukocyte density was decreased in both patient groups (Table 8).

4 Messenger RNA expression and immunohistochemistry (II, III)

Messenger RNA (mRNA) expression of the genes, which showed significant differences between patients and controls, are summarized in Table 9. mRNA expression of Transforming growth factor (*TGF) β2* and Caveolin1 *(CAV1)* were similarly increased both during and after weaning off PN, while expression of Tumor necrosis factor *(TNF)*

was increased only after weaning of PN. mRNA expression of Mucin 2 (*MUC2)* and gene encoded membrane glucose transporter (GLUT1) *SLC2A1* were increased, whereas Claudin 1 encoded gene *CLDN1* expression and NLR family Caspase Recruitment Domain (CARD) containing 4 inflammasome encoded gene (*NLRC4)* expression was decreased during PN dependency (study II). In PN-dependent patients, mucin-2 immunohistochemistry (II, Fig.1, E, F) showed an increased amount of villus goblet cells in relation to matched controls [median (IQR) 0.049 goblet cells/µm (0.044-0.072) vs 0.042 (0.039-0.043), P =0.028], For caveolin-1 (II, Fig 1, G, H), no differences were found between patients and controls in the staining of endothelial and smooth muscle cells. The duration of PN was negatively associated with Marker of proliferation Ki-67 (*MKI67)* expression (r=-0.709, P=0.022) and intraepithelial leukocyte density (r=-0.791, P=0.001).

Following weaning off PN in study III, caveolin 1 immunohistochemistry revealed comparable staining in endothelial and smooth muscle cells in patients and controls (data not shown). *CAV1* expression was positively associated with epithelial leukocyte density (r=0.636, P=0.026). There were no significant differences in the number of MUC2 positive villus goblet cells between patients and controls (data not shown).

5 Effect of small bowel dilatation (III)

SBDR was normal [median (IQR) 1.4 (1.3-1.7)] in 18 patients and pathological [2.4 (2.2-3.4), P<0.001] in nine (Table 5). The ages of patients matched in these two groups [median (IQR) 5.5 (3.0-14.4) vs 2.7 (1.1-10.9) years, P=0.136]. Compared to patients with normal SBDR, pathological dilatation was associated with notably decreased mRNA expression of *IL6* [median (IQR) 0.62 (0.45-0.70) vs 1.85 (0.87-3.10), P=0.008] and *IL18* [0.82 (0.65-0.89) vs 1.24 (0.92-1.48), P=0.01]. *IL6* expression was negatively correlated with SBDR (r=-0.609, P=0.004). Patients with an undilated small bowel showed significantly deeper crypts when compared to patients with dilated small bowels [median IQR) 306 (251-337) vs 246 (210-285) µm, P=0.045] and, in addition, SBDR was negatively associated with crypt depth (r=-0.609, P=0.004). Small bowel diameter showed negative correlation with mRNA expression of proliferation regulating genes

PCNA and *MKI67* (P=0.046 and P=0.002, respectively), providing further evidence that dilatation and crypt homeostasis are linked.

6 Effect of absent ileocecal valve (II, III)

During PN (study II), the absence of an ileocecal valve (ICV) was associated with a lower intraepithelial leukocyte density [median (IQR) 0.02 (0.01-0.03) vs 0.04 (0.03-0.04), P=0.002]. In addition, following weaning off PN (study III), the absence of ICV tended to associate with a broader villi [median (IQR) 305 (263-352) vs. 252 (218-325) μm, P=0.09) and higher intraepithelial leukocyte density [0.03 (0.02-0.04) vs 0.02 (0.01-0.03, P=0.061]. In these patients, absence of ICV was associated with higher mRNA expression of inflammation and epithelial permeability mediating genes *TLR4*, *TGFB1* and *HP*, apoptosis regulating genes *NAIP* and *NLRP1,* and peptide transporter gene *SLC15A1*,when compared to patients with preserved ICV (study III, Figure 5).

Table 8. Morphology, proliferation, apoptosis and inflammation in patients and controls (studies II, III)

	Study II		Study III	
Variable	Patients on PN n=14	Controls n=6	Patients weaned-off PN n=33	Controls n=12
Villus heigth (μm)	597 (422-672)	670 (601-851)	732 (615-861)	810 (665-946)
Villus width (μm)	301 (264-361)	278 (239-367)	285 (232-338)	315 (264-362)
Crypt depth (μm)	258 (229-282)	307 (248-381)	283 (226-320)	273 (228-360)
Crypt proliferation index (%)	57 (37-68)	49 (19-59)	0.68 (0.55-0.79)	0.67 (0.47-0.84)
Villus proliferation index (%)	2.65 (1.21-11.54)	3.42 (0.12-11.80)	0.04 (0.02-0.07)	0.04 (0.01-0.08)
Proliferation grade (1-3)	1.3 (1.0-2.0)	1.5 (1.0-1.8)	1.3 (1.0-1.7)	1.5 (1.0-1.9)
Apoptotic index (per 10 crypts)	0.0 (0.0-0.2)	0.0 (0.0-0.6)	0.1 (0.0-0.5)	0.1 (0.0-0.6)
Lamina propria inflammation (1-4)	2.0 (1.7-2.7)	1.5 (1.3-2.5)	2.0 (1.7-2.7) **	1.5 (1.3-2.0)
Intraepithelial leukocytes (%)	0.03 (0.02-0.04)*	0.04 (0.03-0.16)	0.02 (0.01-0.03) ***	0.04 (0.03-0.13)

Data expressed as median and IQR. Comparisons between patients and controls in each study made using Mann Whitney U test. II, study II. III, study III * P=0.039 vs controls. P= 0.035 vs controls (II). **P=0.033 vs controls, ***P<0.001 vs controls (III). PN, parenteral nutrition.

Table 9. Significantly altered mRNA expression of genes between patients and controls (studies II, III).

	Study II			Study III		
	Patients on PN n=11	Controls n=6	P-value	Weaned-off PN n=25	Controls n=12	P-value
	Inflammation					
TNF	1.32 (0.92–1.31)	1.19 (0.86–1.49)	0.615	1.50 (0.90-2.02)	1.02 (0.61-1.43)	**0.027**
TGFβ2	1.60 (1.29-2.15)	1.08 (0.70-1.40)	**0.035**	1.49 (1.12-1.83)	1.02 (0.77-1.28)	**0.006**
CAV1	1.21 (1.10-1.33)	0.94 (0.86-1.01)	**0.016**	1.31 (1.05-1.46)	0.96 (0.91-1.05)	**0.001**
	Epithelial barrier function					
HPR	0.73 (0.56-0.84)	1.10 (0.99-1.22)	**0.012**	0.81 (0.62-0.99)	1.10 (0.93-1.27)	**0.006**
MUC2	1.56 (1.33-1.84)	1.07 (0.79-1.30)	**0.044**	1.26 (0.79-1.54)	1.11 (0.77-1.54)	0.795
NLRC4	0.86 (0.73-0.94)	1.08 (0.92-1.26)	**0.021**	1.02 (0.75-1.20)	1.02 (0.85-1.23)	0.673
	Nutrient transport					
SLC2A1	1.85 (1.37-2.32)	1.18 (0.66-1.49)	**0.035**	1.33 (0.74-1.76)	1.13 (0.72-1.21)	0.673

Data expressed as mean and IQR. Comparisons between patients and controls in each study made using Mann Whitney U test. EN, enteral nutrition; PN, parenteral nutrition.

DISCUSSION

1 Disaccharidase activities and inflammation (I)

Evaluation of disaccharidases activity revealed that maltase and sucrase activities in patients currently receiving PN were significantly lower than in controls or patients who had achieved enteral autonomy after weaning off PN. Additionally, after discontinuation of PN, disaccharidase activities were comparable to matched controls. Twenty-two percent of the biopsies of PN dependent patients showed inflammation and 30% showed abnormalities in duodenal histology; in these patients, the longer the remaining length of the colon, the higher the activities of maltase and lactase. After weaning off PN, the shorter the length of the remaining small bowel, the higher maltase and sucrase activities were. Maltase and sucrase activities were also positively associated with the post-resectional time, but not age of the patients.

Previous literature reports positive associations between crypt proliferation, mucosal hypertrophy and disaccharidase activities [20,183,184]. TPN was associated with notably lower protein synthesis, while disaccharidase activities were reported to be at similar levels to an unresected small bowel [184]. Petersen *et al*. investigated the effect of TPN, with or without GLP-2 treatment, and enteral nutrition on (unresected) small intestines of premature piglets. They concluded that enteral nutrition and GLP-2-delivery enhance bowel growth but display different effects on small intestinal enzyme activities and function. [185] In accordance with the present findings, prolonged TPN in SBS children has been reported to result in focal villous atrophy, decreased epithelial cell proliferation in the duodenum and lower disaccharidase activities [135].

All PN-dependent patients in this study received different amounts of enteral nutrition in addition to PN, which is likely to promote mucosal health and intestinal adaptation [104,136,137]. These patients were also more likely to have a shorter colon and this associated negatively with disaccharidase activities. One explanation for this negative association could be the reduced amount of L-cells, which are mostly located in the distal ileum and proximal colon and are responsible for the release of GLP-2 [123,124]. This

highlights the important role of the large intestine during intestinal adaptation in SBS children.

PN dependence in this study was associated with increased mucosal inflammation. PN is thought to be related to intestinal bacterial overgrowth and dysbiosis, which may lead to increased mucosal permeability and inflammation [79,85]. In this study, PN seemed to be associated with both inflammation and decreased disaccharidase activities, suggesting impaired intestinal adaptation in these patients. Patients who had achieved enteral autonomy showed higher disaccharidase activities after a longer post-resectional period. It is important to note that no relationship between age and disaccharidase activities were found either in patients or controls, suggesting that higher enzyme activity occurred due to bowel adaptation.

Taken together, current PN-delivery was related to lower disaccharidase activities and increased inflammation, which could negatively affect mucosal function, nutrient absorption and intestinal adaptation. Inflammation, microbial dysbiosis, reduced disaccharidase activities and their mRNA expression in the gut have been previously reported to be linked with each other [186]. A shorter colon in PN-dependent patients (study I) was associated with lower disaccharidase activities. Meanwhile, weaned-off patients (study I) showed that a shorter remaining small bowel related to higher disaccharidase activities. Whether this occurred due to enteral nutrition remained unclear. Further investigation addressing mucosal duodenal morphology is needed as this is novel information and patients' remaining anatomy and underlying diagnosis, as well as medical history, vary between patients. In a recent study, children with SBS (n=8) have shown generally lower or moderate activity of brush border enzymes. Patients in this study also tended to show heterogeneous variety in basic characteristics, nutritional status and anatomical details and these make it difficult to conclude which factors are more important for disaccharidase activity. [20] In weaned-off patients (study I), the longer was post-resectional time, the higher disaccharidase activities were, which may be explained by longer adaptation time and stimulation of enteral nutrition rather than an increase in age. We suggest that low amounts of enteral nutrition together with the remaining bowel anatomy are one of the intestinal adaptation mechanisms.

2 Morphology, proliferation, apoptosis and inflammation (II, III)

In these studies, against our hypotheses, SBS patients on current PN (study II) and after weaning-off PN (study III) showed similar results with matched controls in evaluations of mucosal morphology, proliferation and apoptosis. In contrast with our study, McDuffie *et al*. reported a significant increase in villous height and crypt depth of proximal small intestines in neonates 74±13 days after small bowel resection. The small bowel resection was relatively minor (16 cm) and 92% of participants had weaned off PN at the time of discharge. [187] Rossi *et al*., however, reported minor villous hypoplasia during prolonged TPN in SBS children who were not able to tolerate any oral feeds [135]. Enteral nutrition is known to activate GLP-2 secretion [123,124]. Moreover, exogenous GLP-2 as a treatment in SBS may be associated with villous hyperplasia in the duodenum [126]. Patients in our study had short remaining small bowels of median 20% of expected in PN-dependent patients and 29% of expected in weaned off patients. The median proportion of the remaining colon was 67% and 80%, and ICV was absent in 57% and 52% of the patients, respectively, and only patients on current PN had endostomy (36% of cases). The loss of the proximal large, and most of the distal small intestine may reflect the actual loss of L-cells required for GLP-2-secretion and may explain the missing mucosal hyperplasia despite supplemental enteral feeds in PN dependent patients [107,125]. In a previous study, serum GLP-2 levels correlated negatively with the remaining small intestinal length and the percentage of daily parenteral energy supply. In addition, colonic continuity was associated with increased levels of serum GLP-2. [125] Among weaned-off patients in study III, those with a remaining small bowel length <20% of expected had significantly higher villous length when compared to patients with longer remaining small bowel. Taken together, a shorter small bowel accompanied with longer villi in weaned-off patients may reflect more active adaptation. These results highlight the importance of the remaining bowel anatomy on the intestinal adaptation process.

During studies II and III, we wanted to explore the proportion of patients receiving antimicrobial therapy as it may affect results regarding mucosal inflammation. Patients in our study currently on PN showed similar levels of inflammation in villi lamina propria compared to controls. A previous study presented by Pichler *et al*. showed that nearly half

(five of twelve) of pediatric SBS patients and the majority (eight of thirteen) of patients with dysmotility disorder on PN showed histological small and/or large intestinal mucosal inflammation [87]. The study did not reveal a possible use of antimicrobial therapy in these [87]. However, in our study, patients seemed to have increased inflammation of the lamina propria only after achieving enteral autonomy in comparison with controls and, at the same time, showed molecular signs of inflammation due to elevated levels of mRNA expression of *TNF* and *TGFβ2.* Among PN dependent patients, 86%, and among weaned-off patients only 32%, received antibiotics within time period of six (study II) and two (study III) months before the biopsy, either orally or parenterally. Based on our studies and previous literature, the increased inflammation of the lamina propria seen in weaned-off patients and the reduced inflammation seen in PN dependent SBS patients may be linked to the use of antimicrobial therapy. This may further alter the intestinal microbiota and result in decreased bacterial overgrowth and altered mRNA expression of proinflammatory cytokines. [188-191] Meanwhile, upregulation of *TGFβ2* could be explained by possible immunoprotective respond [192].

3 Messenger RNA expression and immunohistochemistry (II, III)

3.1 Inflammation and cytokines

PN dependent (study II) and weaned-off (study III) patients showed statistically significant increases in mucosal mRNA expression of cytokine producing gene *TGF-β2* and *CAV1,* which encodes plasma membrane protein Caveolin-1 when compared to matched controls [168]. Caveolin-1 is localized into caveolae, plasma membrane invaginations [173]. The crosstalk between Caveolin-1 and TGF-β signaling is reciprocal, very complex and involves several signaling pathways [168,169]. This may contribute to increases of both *CAV1* and *TGF-β2* in patients with SBS observed in this study. TGF-β2 modifies mucosal homeostasis by prolonging or stopping the epithelial cell cycle in the G1-phase, thus reducing proliferation and preventing cells from early apoptosis. [192] Weaned-off patients in our study showed increased *CAV1* expression followed by increased mucosal inflammation when compared to controls. Moreover, patients without an ICV showed higher expression of *CAV1* than patients with an intact ICV. It is therefore suggested that ICV may have an important role in expression of CAV1. As we know, the

ICV prevents bacterial translocation from the large to small intestine and slows transit time. These factors in turn predispose to SIBO, increased mucosal inflammation and permeability, and might contribute to increased expression of *CAV1* [44,81,85].

Increased expression of *TNF* was detected only in weaned-off patients (study III). Gene encodes for tumor necrosis factor (TNF) which is involved in mucosal tight junction through the myosin light chain kinase regulation. TNF acts by inducing occludin removal from caveolar endocytosis, and inhibition of the endocytosis leads to loss of tight junction function. *CLDN1* encodes for tight junction protein claudin-1. [39] Its mRNA expression was elevated in PN-dependent patients (II) in our study. Claudin-1 is a part of tight junction protein complexes which interacts with occludin [39]. The tight junction homeostasis compromises well-balanced crosstalk between caveolae endocytosis and occludin mediated regulation to form transmembrane proteins [39,40]. Exposure to the bacterial toxins may reorganize placement of tight junction proteins and can result in an inability to maintain epithelial barrier function [193].

3.2 Epithelial barrier function

Both patients on current PN (study I) and full enteral nutrition (study III) showed decreased mRNA expression of *HPR*. This gene encodes a haptoglobin-related protein which is localized in the intestinal epithelial cells and was previously described to relate to acute phase proteins and to respond to different types of cytokines [194]. Haptoglobin has been suggested to inhibit the inflammatory responses by several pathways and to result in a decrease of several growth-regulating molecules such as cytokines, growth factors and proteinases [195]. In our study, PN dependent patients (study II), showed decreased mRNA expression of *NLRC4* and increased expression of *MUC2* which was accompanied by an increased amount of villus goblet cells. *NLRC4* encodes NLR family CARD containing 4 inflammasome NLRC4, which triggers caspase-1 mediated cell death called pyroptosis. After caspase activation, the plasma membrane of the cell undergoes pore formation and become permeable, which causes swelling in the cell and eventually results in membrane rupture. NLRC4 is activated by intracellular exposure to specific bacterial proteins and works as a part of the initial immune system. [167] *MUC2* encodes Mucin2 (MUC2) and is also important part of the protective mechanisms in the intestine

from external pathogens. MUC2 operates in the intestinal epithelium, mainly located and expressed in goblet cells, and is responsible for protective gel layer formation. [37] Knocking out of this gene in mouse previously associated with increased bacterial colonization and intestinal inflammation [196]. Our study II does not reveal why patients on PN expressed increased mRNA expression and immunohistochemistry staining for *MUC2*/MUC2. However, several genes (study II) involved in intestinal barrier function *(HPR, MUC2, NLRC4*) seemed to be slightly, but significantly, altered. This may reflect disturbed tight junction homeostasis followed by hampered protection against intraluminal bacterial exposure. In addition, the duration of PN (median 1.4 years) in PN dependent patients (study II) was negatively correlated with the expression of Marker of proliferation Ki-67 (*MKI67)*, this may reflect a negative association between prolonged PN and enterocyte proliferation. *MKI67* encodes Ki-67 protein, which is a well-known marker to recognize cells in the proliferative stage [175].

3.3 Nutrient transport

In our studies, only PN dependent patients (study II), and not weaned-off patients (study III), showed slightly, but significantly, increased mRNA expression of *SLC2A1,* which encodes the plasma membrane glucose transporter 1 [181]. It is one of the SLC2A1-family glucose transporters participating in and maintaining glucose homeostasis, and is expressed in almost every tissue [181]. The gene encoding sodium-dependent glucose transporter (SLGT1) *SLC5A1* showed similar results between patients (studies II, III) and matched controls. Moreover, similar results between patients and matched controls (studies II, III) were observed in mRNA expression of genes maintaining sterol (*ABDG5/8, NPC1L1*), fatty-acid (*FABP2, SLC27A4*), and peptide (*SLC15A1*) absorption. Plasma membrane transporter GLUT1 expression is dependent on glucose metabolism of the tissue and the increased level in our patients (study II) may not reflect the improved glucose metabolism, leaving the meaning of this finding unclear [181].

4 Effect of absent ileocecal valve (study III)

In study III, the mRNA expression of genes related to inflammation (*TLR4, TGFβ1* and *CAV1)*, maintenance of epithelial barrier function (*HP*), apoptosis (NLR family apoptosis

inhibitory protein encoding gene *NAIP* and NLR family pyrin domain containing 1 inflammasome encoding gene *NLRP1)* and peptide transport 1 encoding gene *SLC15A1* were significantly increased in patients without an ICV when compare to patients with an intact ICV.

The role of Caveolin-1 is discussed earlier in chapter 3.1 – Inflammation and cytokines. The toll-like receptor (TLR) family participates in the regulation of antimicrobial defense and TLR4 works as a lipopolysaccharide receptor, participating in anti-inflammatory response [165]. In a rat-SBS model, resection of the bowel resulted in an increased incidence of bacterial translocation to the lymph nodes, liver, portal and peripheral blood when compare to sham-operated rats. These findings were accompanied by *TLR4* up-regulation and a higher density of TLR4 positively stained cells in the remaining small bowel. In addition, *Escherichia Coli* from the proteobacteria group was the main pathogen in bacterial translocation infections of intestinal origin. [197] Proteobacteria are seen to be overabundant in pediatric IF patients and is responsible for producing lipopolysaccharides, which in turn activate TLR4 pathway [82,197,198]. Meanwhile, TGFβ, an immunoregulatory cytokine, is produced inter alia by intestinal epithelial cells and plays a significant role in inflammatory response [199]. TGFβ-1 production is activated, for instance, by bacteria, viruses, cytokines and apoptotic cells and may play an important role in the inhibition of intestinal inflammation [200].

NAIP is a protein coding gene and belongs to the NLR Apoptosis Inhibitory Protein Family, which is divided into two subgroups based on their functions: the CARD and the pyrin domain (PYD). The PYD includes NLRs, for instance NLRP1, NLRP2 and NLRP, by which caspase-1-mediated binding and activation is possible. [201] Caspase-1 activation is related to pyroptotic cell death and is a reaction to the extracellular pathogens in the intestine [167,201]. *NAIP* relates to antiapoptotic functions as well and is important in the regulation of tissue homeostasis [201].

SLC15A1 encodes the enterocyte plasma membrane peptide transporter1, which is related to the transport of di- and tripeptides and its expression on cell membrane is highly dependent on dietary protein supply [182]. In this study (III), patients were all weaned

off PN and the exact information about the quality of nutrition is not available. The reason for the higher expression of this gene in patients without an ICV remains unclear.

The upregulation of several genes related to inflammation, apoptosis and epithelial barrier function in weaned-off patients (study III) without an ICV when compared to patients with an ICV might reflect the importance of this anatomical intestinal valve in the regulation of mucosal homeostasis.

5 Effect of small bowel dilatation

In study III, pathological small bowel dilatation was associated with shorter crypts and decreased mRNA expression of cytokines encoding genes *IL6* and *IL18*. SBDR was inversely correlated with *IL6* expression and crypt depth, as did the measured diameter with mRNA expression of proliferation related genes Proliferating cell nuclear antigen (*PCNA)* and *MKI67*. In murine models, IL6 stimulates epithelial cell proliferation during intestinal inflammation and participates in epithelial repair after intestinal injury [202,203]. IL6 signaling in crypts activates the pSTAT3 pathway in Paneth cells, increasing the number of IL6 receptors. Blocking IL6 receptors (*in vivo*) in Lgr5EGFP mice resulted in a significant reduction of the Lgr5EGFP cells per crypt, the amount of crypt nuclei, crypt depth, and villus height. [204] *IL18* gene encodes the proinflammatory cytokine IL-18, which was shown to be able to modulate tight junction proteins and induce apoptosis, eventually impairing intestinal mucosal integrity after mucosal injury in rats [205]. *PCNA* encodes the protein called cofactor of DNA polymerase delta, which is connected to cell proliferation and was reported to relate to crypt elongation in pigs [206]. Taken together, in our study III, excessive small bowel dilatation was related to shorter crypts and impaired expression of cell proliferative genes *IL6, PCNA* and *MKI67*, while expression of *IL18* was decreased, suggesting that disturbed crypt homeostasis may closely relate to pathological small bowel dilatation.

6 Strength and limitations of this study

In all studies (I, II, III) many patients had no representative biopsies for microscopical evaluation. Seventeen of the 97 specimens from patients and six of 43 in controls were not suitable for the evaluation of morphology, proliferation, apoptosis and inflammation. The data concerning presence or absence of inflammation, or abnormalities in duodenal samples (I), was collected from general, not structured, reports of pathologists, which is a clear weakness of the study. The patients in the first study (I) represented the pediatric IF population well considering the distribution of diagnoses and the number of the patients (Table 2) for a single center study. In addition, the number of controls was considered good (0.7 controls per patient) with the disaccharidase levels in line with previous reports in children with upper-abdominal symptoms [149,207,208]. A clear limitation of studies I and II was the limited number of SBS patients. In addition, the number of control individuals in studies II and III remained low (0.4 controls per patient for both studies). However, we were able to conduct detailed comparative analysis of adaptation-related mucosal changes in all controls. These studies were retrospective, based on hypotheses and included multiple comparisons, which increases the possibility of false positive findings. We emphasize that the results represent associations rather than proven causal relationships. Finally, this study provides novel information addressing duodenal mucosal homeostasis in children with IF (I) and SBS (I-III).

CONCLUSIONS

To conclude, we evaluated disaccharidase activities and assessed the level of histological inflammation in the duodenum in pediatric IF patients on PN and after weaning off PN. This work (study I) showed that maltase and sucrase activities tended to be lower, and mucosal inflammation increased, in patients on current PN when compared to patients who had weaned off PN and matched controls. The extent of bowel resection and time after weaning off PN was associated with increasing disaccharidase activities amongst patients, suggesting that disaccharidases might be one of the mechanisms promoting functional intestinal adaptation.

We evaluated duodenal mucosal microarchitecture, proliferation, apoptosis, inflammation, and epithelial-barrier function in PN dependent (study II) and weaned-off patients (study III) in relation to matched controls. Our findings suggest that duodenal mucosal hyperplasia has a limited role in post-resectional intestinal adaptation in children with SBS. Despite weaning off PN, patients (study III) showed molecular signs of mucosal inflammation and altered regulation of epithelial permeability in the duodenum.

We measured small bowel dilatation and assessed its relation to mucosal microarchitecture, proliferation, apoptosis or inflammation in weaned-off patients (study III). The main findings revealed that excessive dilatation of the remaining small bowel was accompanied by impaired duodenal crypt homeostasis, and an absent ICV with altered mRNA expression of genes regulating mucosal inflammation and permeability.

Overall, these findings provide novel information addressing duodenal microarchitecture and duodenal mucosal homeostasis in pediatric SBS patients. Further studies are needed to explore mechanisms and the functional significance of our findings.

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